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MELANIN FORMATION IN COLOR VARIETIES OF THE MEDAKA (*ORYZIAS LATIPES*)[1)2)]

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INTRODUCTION

There are a number of color varieties of a cyprinodont fish, the medaka (*Oryzias latipes*). The chromatophores responsible for body color of this fish are melanophores, xanthophores, and guanophores. The brown chromatophore described by GOODRICH (1927) seems to correspond to our guanophore which is brown by transmitted light and white by reflected light.[3)] The inheritance of body color of this fish has been studied by ISHIHARA (1917) and AIDA (1921). Excellent work by the latter revealed that the various color effects are referred to the action of genes. When the genes are homozygous the color varieties are assigned as follows: brown (wild type) *BB, RR*; blue *BB, rr*; orange-red *bb, RR*; white *bb, rr*; variegated orange-red *B'B', RR*; and variegated white *B'B', rr*. The triple allelomorphic genes *B, B'*, and *b* control the formation of the melanin in the melanophore and are autosomal. The gene *B*, which is dominant in effect to both *B'* and *b*, permits the full formation of the melanin (Fig. 1). *B'* is dominant in effect to *b* and causes variegation. There are pigmented and non-pigmented area in the genotypes *B'B'* and *B'b* producing the mottled appearance. In overall appearance, pigmented areas of the varieties with *B'* are irregularly distributed. There is, however, a tendency to form irregular transversal stripes. In the orange-red (*bb, RR* or *bb, Rr*) and white (*bb, rr*) the formation of the melanin is so slight that the melanophores

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1) Partly read before the 24th Annual Meeting of Zoological Society of Japan held at Kyoto on November 1-4th in 1953 and the résumé was published in Zool. Mag. (Tokyo), *63*, 169 (1954).

2) This is a part of the "Phenogenetic studies on color varieties of the medaka (*Oryzias latipes*)".

3) This type of chromatophore has been designated as "guanophore" by Japanese researchers although chemical nature of its white pigment (whether guanin or not) has not yet been clarified.

are usually invisible in an expanded condition (Figs. 2 and 3). As pointed out by GOODRICH (1927), however, the presence of a small amount of melanin in the "colorless" melanophores can be demonstrated by congregating the granules of the melanin through treatment with adrenalin.

The development of chromatophores in this species was described by GOODRICH (1927) wherein special reference was made to the characters controlled by genes B, B', and b. According to him, the number of melanophores is the same in all these phenotypes; however, they differ in their melanin-forming potency. OKA (1930, 1931) reached the conclusion that B, B', and b cause the grade of development of melanoblast with respect to cell size and the degree of the production of melanin in that order. We should like to call melanophores of the adult in the light-colored varieties (bR and br) "colorless" or "amelanotic" melanophores. By the term amelanotic melanophores we mean those melanophores which contain either very small amounts of melanin or no melanin at all. It should be noted in this connection that the light-colored varieties (bR and br) are not true albinos since their eyes and peritoneum are black; however, they may be regarded as partial or semi-albinos.

The genes R and r govern the deposition of an orange-red pigment (carotenoid) in xanthophores and are sex-linked. According to GOODRICH *et al.* (1941) the carotenoid in this fish is lutein (xanthophyll).

In 1951, EIZI NAKABORI, a fish fancier, collected a pair of gray medaka from a wild population in a certain region on the northern outskirts of Kyoto. AIDA (unpublished) performed breeding experiments with these fish and found that they have a color interferer (*ci*) in the duplex condition. He elucidated that the *ci* is a Mendelian recessive and is independent from other color genes. The F_1 of the gray (*cici*, BB, RR) mated with the white ($++$, bb, rr) are brown ($+ci$, Bb, Rr). According to him (personal communication to T.Y.), the phenotype *ciBR* is "pale blue" because of the paleness of both the orange-red and black colors, reduction of the former color being more profound than that of the latter. The phenotype may preferably be called gray. The number of guanophores in this phenotype is significantly increased according to a study made in our laboratory. An account on the effect of *ci* on chromatophores will be presented

elsewhere.

Biochemical genetics of the medaka has not progressed further since GOODRICH (1933) demonstrated the presence of dopa oxidase in "colorless" melanophores. Dopa, however, is an immediate intermediate produced from tyrosine which is believed to be the precursor of the melanin. The chief obstacle to the study of hereditary variation in the tyrosinase system has been the difficulty to demonstrate the enzyme in vertebrates. ROTHMAN *et al.* (1946) first demonstrated the presence of an inhibitor of tyrosinase in the human skin and the removal of this inhibitor by iodoacetamide. Their findings encouraged us to investigate the mechanism of the formation of the melanin in color varieties of the medaka.

The present study is a first step in the approach to the problem of pigment formation as controlled by genes. Special reference is made to the detection of amelanotic melanophores and the presence of tyrosinase in the light-colored varieties (*bR* and *br*).

We want to express our deep gratitude to Dr. SEIZO KATSUNUMA, the former President of Nagoya University, for his interest in this project and for his continued encouragement during the present study.

MATERIAL AND METHOD

The material used in the present study are the scales with the skin removed from the adults of the following varieties: brown (wild) *BR*, blue *Br*, orange-red *bR*, white *br*, and variegated red *B'R*. The fish were first anesthetized with 0.035% chloretone. The scales located in certain regions on the trunk were removed with forceps and immersed in Ringer's solution for medaka composed of M/7.5 NaCl 100 parts + M/7.5 KCl 2.0 parts + M/11 $CaCl_2$ 2.1 parts (pH 7.3 being adjusted by N/10 $NaHCO_3$) as formulated by YAMAMOTO (1939, 1944). Isolated scales were allowed to stand in the solution for at least one-half hour by which time the melanophores expand fully.

For the dopa oxidase reaction, isolated scales were incubated in an equal-volume mixture of (1) 0.1% dopa (3, 4-dihydroxyphenyl-*l*-alanine, Eastman Kodak Co.) in M/7.5 NaCl solution and (2) M/10 phosphate buffer (pH 6.8 and 7.3) for four hours at 37° C. To avoid autoxidation, dopa solution was prepared just before its use and the mixture was renewed after two hours to prevent the formation of an excessive deposit of dopa melanin by autoxidation.

For the tyrosinase reaction, the incubation mixture of the following composition was first used.

(A) Tyrosine (0.1%) + $NaHCO_3$ (0.04%) in M/7.5 NaCl...6 parts
(B) M/10 phosphate buffer (pH 7.3)....................3 parts
(C) M/10 iodoacetamide solution.......................1 part.

Isolated scales were incubated in the mixed solution for 24 hours at 37° C. Later it was found that other sulfhydryl reagents such as *p*-chloromercuribenzoic acid (M/100), monoiodoacetic acid (M/10), and even a non-sulfhydryl reagent (0.05% guanofuracin) could be used successfully as a substitute for the (C) solution. We also have succeeded in detecting tyrosinase by pretreating the scales with a number of agents (cf. under Results) and then immersing these scales in the mixture of (A) and (B) only. In such experiments, the addition of penicillin or streptomycin (final concentration: 50–100 i.u./ml.) to the tyrosine solution is necessary in order to guard against bacterial and fungial infection during the incubation period.

RESULTS

Dopa oxidase reaction

In recent years, the dopa oxidase reaction has been utilized to detect the melanophores in non-pigmented skins (GOODRICH 1933, RUSSEL 1939, GINSBERG 1944, RUSSEL and RUSSEL 1949). To verify the presence of dopa oxidase, the scales taken from orange-red, white, and variegated fishes are incubated in a modified dopa solution. The majority of the melanophores in the incubated scales appear in a stellate form having a great deal of dopa melanin but the contour of the melanophores is not so clear because of an accumulation of dopa melanin which is formed by autoxidation upon the surface of the scales.

The scales, fixed in a 10% formalin solution for an hour, show almost no dopa oxidase activity. The formation of the melanin in the incubated melanophores is completely inhibited by the addition of KCN or diethylthiocarbamate, the final concentration of both being M/500. With incubated scales, after heating for five minutes at 80° C, the melanin which has been formed does not become localized in the melanophores but is deposited uniformly on the surface of the scales.

These facts prove the presence of dopa oxidase in the amelanotic melanophores of the medaka.

Tyrosinase reaction

In the medaka, as is common with all vertebrates, it has been quite difficult to prove the presence of tyrosinase. No melanin is formed when the scales of orange-red and white fishes are incubated in a tyrosine solution, buffered to pH 7.3, for more than 48 hours at 37° C.

First, the presence of tyrosinase was successfully demonstrated by adding a sulfhydryl blocking reagent, iodoacetamide, to a tyrosine solution. By this procedure, the amelanotic melanophores, otherwise undetected, are revealed as well-defined melanophores containing numerous granules of melanin (Fig. 4). These melanophores are in a considerably expanded reticulate state as those of the brown and blue varieties (cf. Fig. 1).

In the variegated variety (*B'R*), the scales from the dark area normally has fully developed as well as partially developed melanophores varying from dots to splotches which are, however, more highly differentiated than the melanoblasts. The incubated scales taken from the light area indicate that they have many well-developed and undeveloped amelanotic melanophores.

Monoiodoacetic acid can be used instead of iodoacetamide, though its action is weaker. The powerful sulfhydryl specific poison, *p*-chloromercuribenzoic acid, is effective for detecting the presence of the tyrosinase system in the amelanotic melanophores in a concentration as low as 10^{-3} M. Higher concentrations of the reagent are less effective for this purpose.

In search for reagents other than the sulfhydryl poisons, the following are found to be effective for the formation of the melanin in the amelanotic melanophores. Guanofuracin (0.05%) can replace the iodoacetamide (C) solution in the incubation mixture. By the end of the incubation period, pigmented melanophores appear with a stellate form in isolated scales from the orange-red variety (Fig. 5). Pretreatment of the scales with chloretone (0.35%), chloroform (sat. sol.), and octyl alcohol (sat. sol.) and subsequent incubation with the tyrosine-buffer solution (A + B) can induce the formation of the melanin in the amelanotic melanophores provided that an antibiotic

(penicillin or streptomycin) is added to the mixture. In these experiments, isolated scales are first treated with one of these reagents for one minute at a low temperature (0° C), then rinsed in distilled water, and finally incubated separately in the tyrosine solution with an antibiotic. The amelanotic melanophores of the orange-red and white varieties turn black by these procedures. They are in a fully expanded state as are those incubated with iodoacetamide (Figs. 6, 8, and 10). Short pretreatment of the scales with acetone (40%), methanol (50%), ethanol (50%), formalin (10%), and urea (M/3), and subsequent incubation in the tyrosine solution is also effective for the pigmentation of the amelanotic melanophores (Figs. 16, 7, 14, 15, and 11). The procedures with this group of reagents need more delicate handling than those with the former group (chloretone etc.). Pretreatment of the scales with warm water (60° C) for one minute can also induce the formation of the melanin in the amelanotic melanophores by subsequent incubation with the tyrosine solution. The majority of the pigmented melanophores resulting from this procedure, however, are in a punctate state (Fig. 12). It is worth noting here that the formation of the melanin sometimes proceeds to some extent without any pretreatment in isolated scales incubated in the tyrosine solution which contains only an antibiotic (penicillin or streptomycin) (Figs. 13 and 9).

Without exogenous tyrosine, the melanin-pigment can not be produced during the incubation in any cases. The formation of the melanin is completely inhibited by the addition of KCN (final concentration M/500) to the incubation mixture. Similar inhibitory results are obtained by phenylthiourea (final conc. M/1000) and diethylthiocarbamate (final conc. M/1000). The scales fixed in a 10% formalin solution for an hour, or heated for several minutes prior to incubation results in inhibition of the formation of the melanin. All these facts support the conclusion that tyrosinase is present in an inactive state in the amelanotic melanophores of the light-colored (*bR* and *br*) and the variegated (*B'R*) varieties of the medaka.

So far we have been concerned with the formation of the melanin in the amelanotic melanophores of the light-colored varieties. Our next concern is the number of the melanophores in these color varieties. Goodrich (1927) concluded that the light-colored varieties (*bR* and *br*) have as many of the melanophores as the dark-colored

variety (*BR*). The present study, as well as a more detailed research performed in our laboratory, does not support this conclusion. Contrary to what we expected, the light-colored varieties with the gene *b* in the duplex condition are characterized by having approximately twice the number of the amelanotic melanophores as those of the dark-colored varieties (*BR* and *Br*). This discrepancy may be attributed to the difference in the methods used for detecting the amelanotic melanophores. Goodrich's conclusion is based on the adrenalin test whereas the method employed in our laboratory is the tyrosine-tyrosinase reaction with iodoacetamide. The adrenalin test congregates small amounts of granules of melanin at the center of the melanophores to make the otherwise invisible melanophores detectable. This method will give a negative result when the amelanotic melanophores are completely free of melanin; therefore, there is a possibility of overlooking the amelanotic melanophores.

Discussion

Raper (1927, 1928, 1932) made it clear that, in the flour beetle (*Tenebrio molitor*), the enzyme tyrosinase catalyzes the oxidation of *l*-tyrosine into dihydroxyphenyl-*l*-alanine (dopa) and the latter is converted into melanin through a chain of reactions. Until the decade of the 1940s, however, the presence of tyrosinase in vertebrate tissues had not been conclusively demonstrated. It has been suspected that vertebrate melanin might be formed by a mechanism different from that operating in plants and invertebrates, *i.e.*, by a specific "dopa oxidase" system (Bloch, 1927). Thus the dopa oxidase reaction has been widely utilized for the detection of melanocytes in non-pigmented skins from mammals (Russel 1939, Ginsburg 1944, Russel and Russel 1949). Lerner and Fitzpatrick (1948) first detected tyrosinase in normal human skin which had been pretreated with ultraviolet radiation. Foster (1951, 1953, 1959) demonstrated the presence of tyrosinase in a young mouse and a fetal guinea pig. These facts led to the view that in vertebrates also tyrosine is the substrate from which melanin is formed. The extraction of tyrosinase from the melanomas of mammals (Hogeboom and Adams 1942, Lerner *et al.* 1949) and fish (Humm and Humm 1959) has been accomplished successfully, but not yet from the normal tissues of adult vertebrates.

The invisible scattered granules of melanin in the amelanotic melanophores congregate at the cell-center by the action of adrenalin. On the basis of this test, GOODRICH (1927) showed that the light-colored varieties (*bR* and *br*) of the medaka owe their apparent absence of melanotic color to a reduction in the amount of the melanin. GOODRICH (1933) inferred that these cells are incapable of producing the normal amount of chromogen although sufficient oxidase is present. His conclusion, however, is based on the dopa oxidase reaction. In the paradise fish, *Marcopodus opercularis*, there is an albinotic variety which is Mendelian recessive to the normal type. On the basis of the criteria with the adrenalin test and the dopa oxidase reaction, GOODRICH and SMITH (1937) concluded that even the amelanotic melanophores are absent in the albino. In this respect, the albinotic paradise fish is in striking contrast to the light-colored varieties of the medaka.

The dopa oxidase reaction, though positive in our experiments, is found to be not so excellent as a practical method for detection of the amelanotic melanophores. Dopa melanin usually deposits itself uniformly on the surface of the isolated scales owing to the autoxidation of the dopa. This situation renders it obscure to observe the dopa melanin formed within the amelanotic melanophores. Moreover, a positive dopa oxidase reaction seems to be not so reliable as proof for the presence of dopa oxidase because the dopa is too easily oxidized by nonspecific substances. HESSELBACH (1951) showed that dopa is autoxidized into melanin by the air or cytochrome oxidase *in vitro*.

The tyrosinase reaction used in the present study affords a more useful and reliable method for the detection of the amelanotic melanophores as well as the evidence for the presence of the oxidase (tyrosinase) because tyrosine is not autoxidizable. By use of this method it is found that the amelanotic melanophores of the light-colored varieties (*bR* and *br*) of the medaka are in a fully developed state with the dendritic processes and have the potency of forming full amounts of the melanin when tyrosine and a certain agent are supplied. The variegated variety ($B'R$), however, is found to have both fully developed and undeveloped melanophores, both of which are either pigmented or amelanotic.

ROTHMAN *et al.* (1946, 1948) found that an aqueous extract of

human epidermis inhibits the oxidation of *l*-tyrosine and dopa. Since this inhibitory effect is counteracted either by iodoacetamide or *p*-chloromercuribenzoic acid, they postulated that the inhibition in normal skin is due to the presence of sulfhydryl compounds. The amelanotic melanophores in our case form full amounts of the melanin in the presence of tyrosine and of sulfhydryl specific poisons. The formation of the melanin in the amelanotic melanophores, however, is induced not only by guanofuracin (a bacteriocide) but also by a short treatment with such agents as chloroform, octyl alcohol, acetone, urea and heat which have been used by BODINE and ALLEN (1938) in the study of the formation of the melanin in insects.

From our findings, it is difficult to draw any conclusion as to the nature of the inhibitor or the inhibitory state. It should be borne in mind that all the agents used in our experiments kill the melanophores as evidenced by their failure of response after the end of incubation. In other words, our experiments are conducted in such a way that the vitality of cells has been lost while the tyrosinase has not yet been denaturated. It is suspected that even those cells incubated in the tyrosine solution with an antibiotic (penicillin or streptomycin) without any pretreatment might be denaturalized before the end of 24 hours at 37° C; therefore, there is a possibility that an inhibitor or inhibitory state is removed in association with the killing of the cells caused by a variety of agents.

To sum up, the amelanotic melanophores in the light-colored varieties (*bR* and *br*) of the medaka are in the state of full development and tyrosinase does exist in an inactive state within them. The inhibitory substance or state can be removed not only by sulfhydryl poisons but by a variety of agents which kill the cells but do not denature tyrosinase. As to the biochemical difference in pigmentation for which the genes *B* and *b* are responsible, further quantitative researches are necessary to elucidate whether they control the production of tyrosine or govern the formation or accumulation of the inhibitory substance.

SUMMARY

1. The formation of the melanin in color varieties of a cyprinodont fish, the medaka (*Oryzias latipes*), which are controlled by

the triple alleles *B*, *B'*, and *b*—where *B* stands for full pigmentation, *B'* for variegation and *b* for deficient formation of the melanin—is studied qualitatively.

2. Methods for detection of the "amelanotic melanophores" in the light-colored varieties (orange-red *bR* and white *br*) with the gene *b* in the duplex condition are presented. The tyrosinase reaction is found to give a more reliable result than the dopa oxidase reaction for this purpose.

3. The light-colored varieties (*bR* and *br*) are found to have the amelanotic melanophores which are as fully developed as those of the dark-colored varieties (brown *BR* and blue *Br*). The variegated orange-red variety (*B'R*), on the other hand, has well-developed as well as undeveloped melanophores, both of which are either pigmented or amelanotic.

4. The number of the amelanotic melanophores in the light-colored varieties is significantly greater than (about twice) that of the melanophores in the dark-colored varieties.

5. Tyrosinase does exist in an inhibited state in the amelanotic melanophores of the light-colored (*bR* and *br*) and variegated (*B'R*) varieties. The inhibitor or inhibited state can be removed not only by sulfhydryl poisons but a variety of other agents.

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(*Manuscript received: 17. Oct., 1960*)

EXPLANATION OF PLATE 2

Magnification of Figures ×70

Figs. 1–3. Normal scales of three color varieties in the medaka.

Fig. 1. The scale of the brown variety (*BR*). Fully developed black melanophores as well as xanthophores are present.

Fig. 2. The scale of the orange-red variety (*bR*). Xanthophores are present, but melanophores are invisible.

Fig. 3. The scale of the white variety (*br*). Both melanophores and xanthophores are invisible.

Figs. 4–8. Tyrosinase test in the scale of the orange-red variety (*bR*).

Fig. 4. Fresh scale incubated with tyrosine in the presence of M/100 iodoacetamide. Amelanotic melanophores are pigmented, their shape and size being similar to those of the black melanophores of the brown variety (*BR*). (cf. Fig. 1).

Fig. 5. Fresh scale incubated with tyrosine in the presence of 0.05% guanofuracin.

Fig. 6. Pretreatment with 0.35% chloretone for one minute at 0° C.

Fig. 7. Pretreatment with 50% methanol for one minute at 0° C.

Fig. 8. Pretreatment with distilled water saturated with chloroform for one minute at 0° C.

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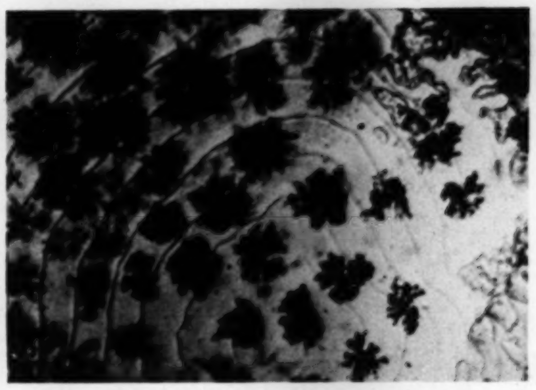

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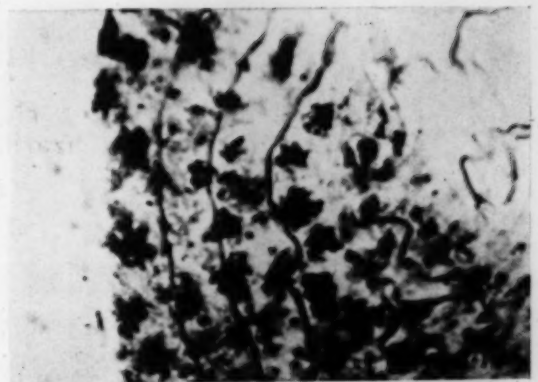

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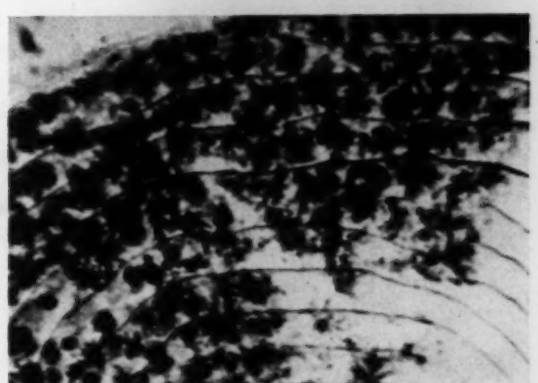

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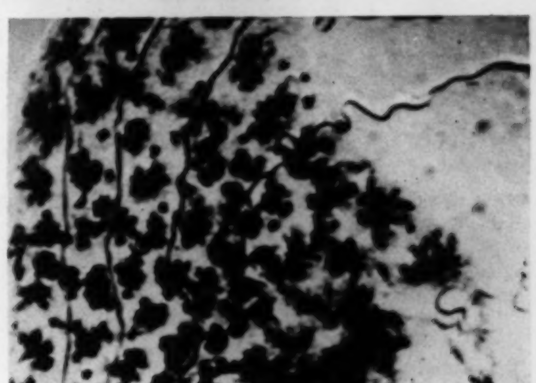

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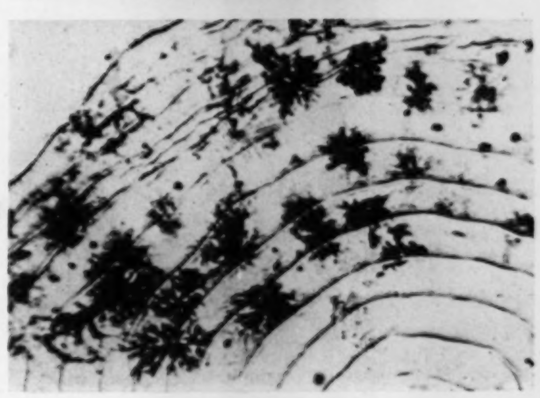


Explanation of Plate 3

Figs. 9–16. Tyrosinase test in the scale of the orange-red variety (***bR***) (Continued)

Fig. 9. Fresh scale incubated with tyrosine in the presence of streptomycin.

Fig. 10. Pretreatment with distilled water saturated with octyl alcohol for one minute at 0° C.

Fig. 11. Pretreatment with M/3 urea for one minute at 0° C.

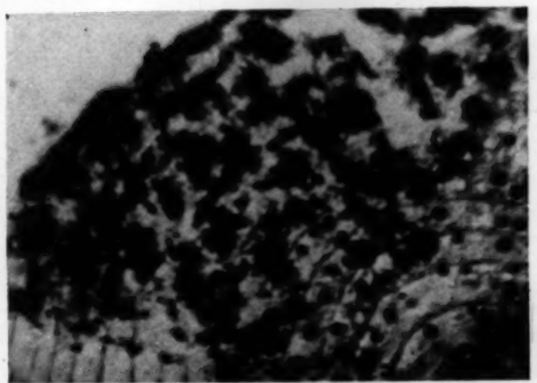
Fig. 12. Pretreatment with distilled water (60° C) for one minute.

Fig. 13. Fresh scale incubated with tyrosine in the presence of penicillin.

Fig. 14. Pretreatment with 50% ethanol for one minute at 0° C.

Fig. 15. Pretreatment with 10% formalin for one minute at 0° C.

Fig. 16. Pretreatment with 40% acetone for one minute at 0° C.



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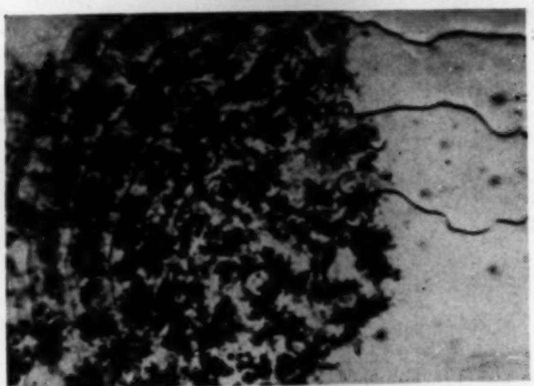

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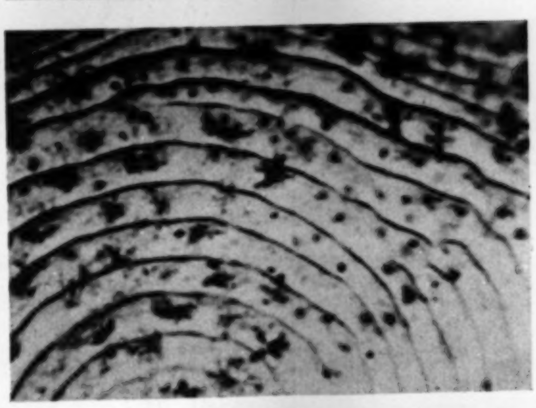

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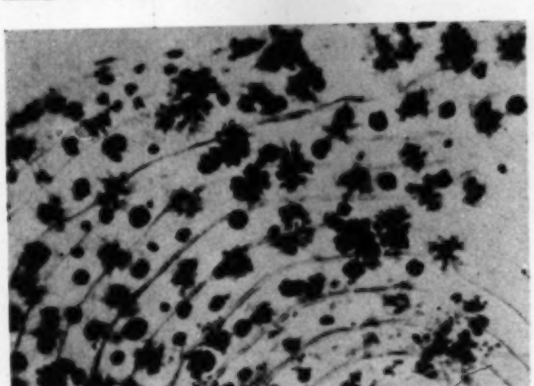

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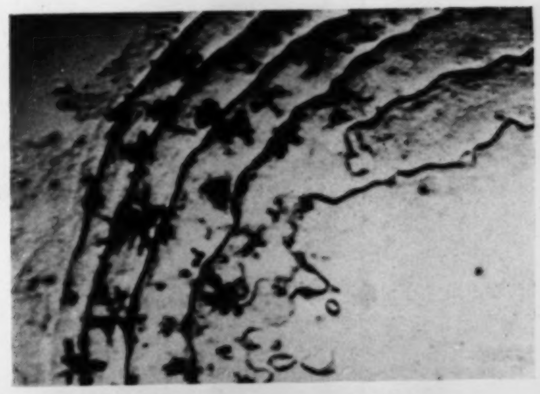

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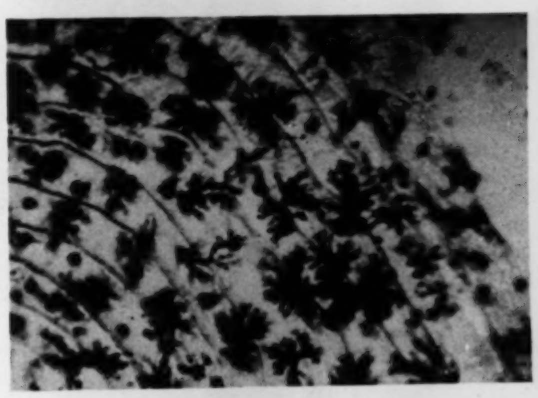

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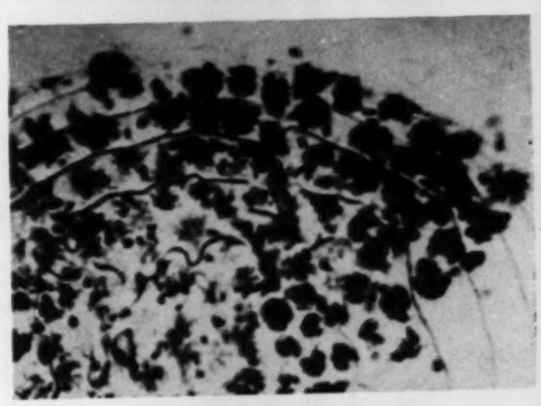

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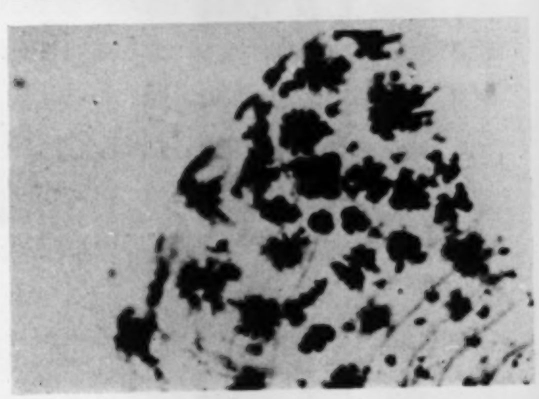

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EMBRYOLOGIA, Vol. 5, No. 4, pp. 347-356 (February, 1961)

A QUANTITATIVE STUDY ON PHENOL OXIDASE OF SKINS IN COLOR VARIETIES OF THE MEDAKA (*ORYZIAS LATIPES*)[1]

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INTRODUCTION

There are some color varieties of the medaka, *Oryzias latipes*. AIDA (1921) demonstrated that the body colors in varieties of the medaka are governed by genes. When homozygous, the color varieties are symbolized by the following constitution: brown (wild type) *BBRR*, orange-red *bbRR*, white *bbrr*, orange-red variegated with black *B'B'RR*, white variegated with black *B'B'rr*. The triple alleles *B*, *B'*, and *b* control the formation of the melanin in the melanophores and are autosomal. The gene *B* permits the full formation of the melanin, *B'* causes variegation and *b* in the duplex condition results in the inhibition of the formation of the melanin. The genes *R* and *r* govern the deposition of a carotenoid in the xanthophores and are sex-linked. For long time only the aforementioned genes have been known to be responsible for body color. AIDA (unpublished) found a color interferer (*ci*) which in the duplex condition results in paleness of the body color. Thus the genotype *ciciBBRR* is gray. As to the origin of the mutant and its genetic behaviour as worked out by AIDA, a brief account is given elsewhere (HISHIDA, TOMITA and YAMAMOTO 1961). Development of Mendelian characters in the medaka has been studied by GOODRICH (1927) and OKA (1931). According to GOODRICH the brown variety (wild type) is characterized

[1] This is a part of the "Phenogenetic studies on the color varieties of the medaka (*Oryzias latipes*)" being carried out under the direction of Prof. TOKI-O YAMAMOTO at the Biological Institute, Faculty of Science, Nagoya University. We express our deep gratitude to Dr. SEIZO KATSUNUMA, the former President of Nagoya University, for his interest in this project and for his continued encouragement during the present study.

by the presence of fully pigmented melanophores, whereas the orange-red and the white varieties have "colorless" melanophores. In this connection it should be noted that these light-colored varieties of the medaka are not albino since their eyes and peritoneum are pigmented. Furthermore, even the amelanotic (colorless) melanophores may have small amounts of the melanin. This fact can be demonstrated by treating them with adrenalin as pointed out by GOODRICH.

Biochemical genetics of the medaka has not progressed further since GOODRICH (1933) demonstrated the presence of dopa oxidase in the amelanotic melanophores of the light-colored varieties. Recent studies on the formation of the melanin have shown that the conversion of tyrosine into melanin in vertebrates is also catalyzed by tyrosinase (HOGEBOOM and ADAMS 1942, LERNER and FITZPATRICK 1948, LERNER 1953, DAWSON and TARPLEY 1951, FOSTER 1951-1959). However, only a few studies have been attempted along this line in fishes (FITZPATRICK *et al.* 1950, HUMM and HUMM 1959). HISHIDA, TOMITA and YAMAMOTO (1961) first demonstrated the presence of the fully developed amelanotic melanophores and of tyrosinase in the light-colored varieties (*bR* and *br*). The present study deals with a quantitative study on the activities of tyrosinase and dopa oxidase in the skins of various color varieties of the medaka.

MATERIAL AND METHOD

The color varieties of the medaka (*Oryzias latipes*) used in the present study were brown (*BR*), orange-red (*bR*), white (*br*), variegated orange-red (*B'R*) and gray (*ciBR*).

Pieces of their skins were dissected and put into cold acetone (below $-5°$ C.) for 30 minutes. The resulting precipitate was collected by filtration, washed with cold acetone and dried under vacuum. The dry material was homogenized in M/50 phosphate buffer (pH 6.8) in a glass homogenizer of the usual type. The homogenate was centrifuged for 10 minutes at a speed of less than 1000 r.p.m. The supernatant was used as the crude enzyme solution. Homogenates of fresh skins also were used, in particular, for measuring the dopa oxidase activity. In this case the homogenate was centrifuged for 10 minutes at 2000 r.p.m., and the supernatant was used as the enzyme solution.

Enzymatic activity was measured by means of a Warburg manometer, and was expressed in terms of the oxygen consumption during the oxidation of tyrosine or dopa at 30° C. A correction for endogenous respiration was made by running a parallel experiment without a substrate. Occasionally, 0.1 ml. of M/10 monoiodoacetic acid or M/10 iodoacetamide was added in order to reduce the endogenous respiration. These agents hardly have any effect on dopa oxidase and tyrosinase activities. The main part of the vessel contained 1 ml. of enzyme and 0.5 ml. of phosphate buffer (pH 6.8). The side arm contained 0.5 ml. of the substrate, while the centre well contained 0.1 ml. of 20% potassium hydroxide. As a substrate 0.5 mg. per vessel of dopa (3, 4-dihydroxyphenyl-*l*-alanine) or *d, l*-tyrosine was used. The former was dissolved in distilled water and the latter in 0.08% sodium carbonate solution.

Substrate specificity was tested on *p*-, *m*-, and *o*-cresols, *p*-chlorophenol, *p*-bromophenol, phenol, 3, 4-dimethylphenol, 3, 5-dimethylphenol, 3, 4-dihydroxybenzoic acid and catechol. Stock solutions of these chemicals were prepared in 0.5%, which were later diluted to the concentrations needed.

Results

Phenol oxidase in the skin of the medaka could oxidize tyrosine and dopa, but it did not oxidize *p*-, *m*-, and *o*-cresols, *p*-chlorophenol, *p*-bromophenol, 3, 4-dimethylphenol, 3, 5-dimethylphenol, 3, 4-dihydroxybenzoic acid, phenol and catechol. The enzymatic activity was inhibited by M/1000 phenylthiourea, M/1000 sodium cyanide and M/1000 diethylthiocarbamate, and was lost by heating the solution for 10 minutes.

The fishes belonging to the orange-red and the white varieties showed a dopa oxidase activity which was about ten times higher than that of the brown and the gray fishes. The tyrosinase activity also was higher in the orange-red variety than in the brown variety. In fact, it is possible that in the brown fish tyrosinase and dopa oxidase activities lack altogether, since when measured, both activities were too low to be significant. The variegated orange-red fish showed almost the same dopa oxidase activity as the orange-red and the white fishes. Consequently, there is no clear relation between

the dopa oxidase activity and the surface area of the unspotted skin in the variegated variety. The results are shown in Figures 1 and 2, and in Tables 1 and 2.

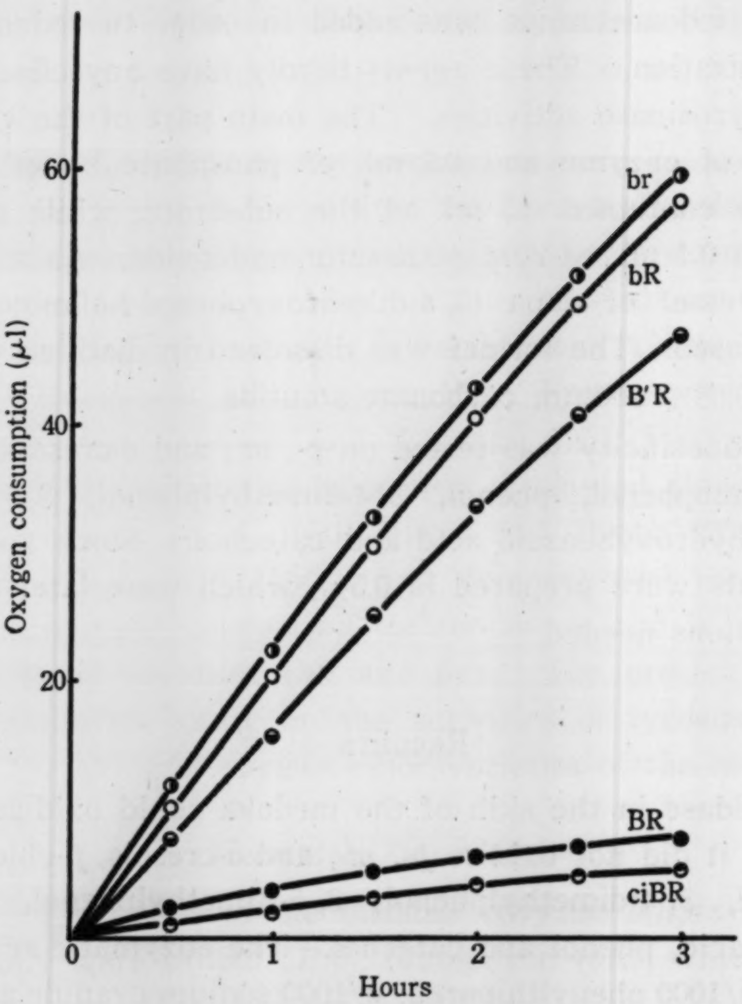

Figure 1. Dopa oxidase activity in the various color varieties, expressed as oxygen consumption per hour per mg. nitrogen of skin extract (endogenous respiration is subtracted). *BR*, the brown variety; *ciBR*, the gray variety; *B'R*, the orange-red variety variegated with black; *bR*, the orange-red variety; *br*, the white variety.

The latent period of the tyrosinase was long, whereas that of the dopa oxidase was short. Blackening of the reaction mixture occurred in parallel with an increase in the oxygen consumption.

For the purpose of comparison, the retinal pigment epithelia

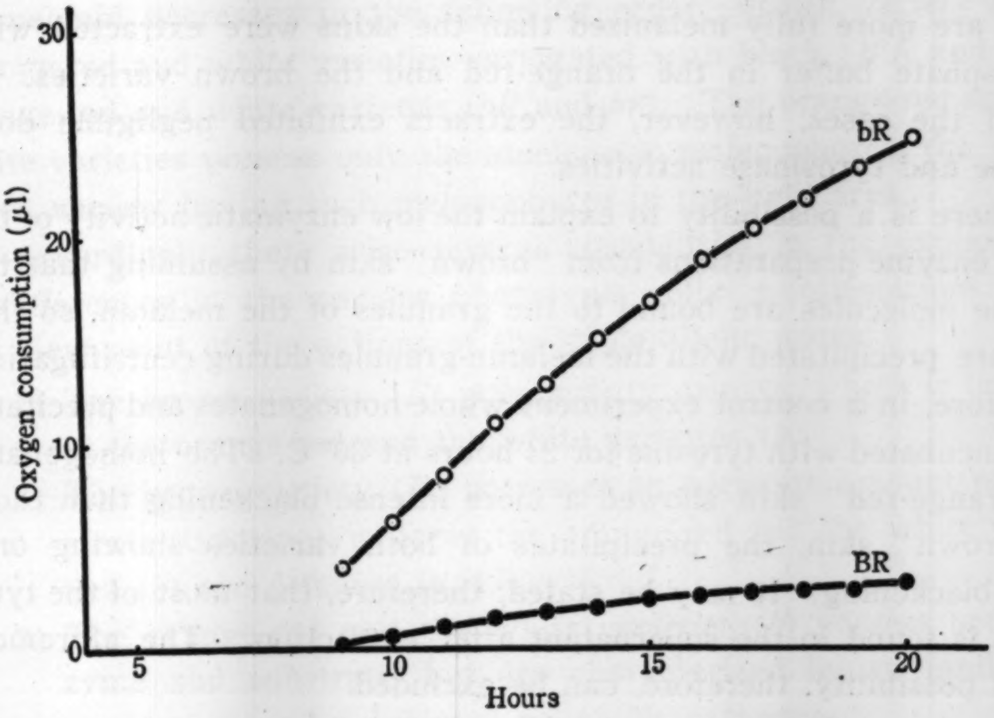

Figure 2. Tyrosinase activity of the brown and the orange-red varieties, expressed as oxygen consumption per mg. nitrogen of skin extract (endogenous respiration is subtracted). *BR*, the brown variety; *bR*, the orange-red variety.

Table 1. Dopa oxidase activity

Activity is expressed as oxygen consumption per hour per mg. nitrogen of skin extract in the presence of dopa (endogenous oxygen consumption is subtracted).

Color varieties		Dopa oxidase activity Range	Average
Brown	(*BR*)	0—4.4	2.4
Gray	(*ciBR*)	0—2.0	1.2
Orange-red	(*bR*)	11.2—26.6	18.6
White	(*br*)	16.0—24.0	19.8
Variegated orange-red	(*B'R*)	11.0—22.0	15.1

Table 2. Tyrosinase activity

Activity is expressed as oxygen consumption per hour per mg. nitrogen of skin extract in the presence of tyrosine (endogenous oxygen consumption is subtracted).

Color varieties		Tyrosinase activity Range	Average
Brown	(*BR*)	0	0
Orange-red	(*bR*)	1.4—1.9	1.7

which are more fully melanized than the skins were extracted with a phosphate buffer in the orange-red and the brown varieties. In any of the cases, however, the extracts exhibited negligible dopa oxidase and tyrosinase activities.

There is a possibility to explain the low enzymatic activity of the crude enzyme preparations from "brown" skin by assuming that the enzyme molecules are bound to the granules of the melanin, so that they are precipitated with the melanin-granules during centrifugation. Therefore, in a control experiment whole homogenates and precitates were incubated with tyrosine for 24 hours at 30° C. The homogenates of "orange-red" skin showed a more intense blackening than those of "brown" skin, the precipitates of both varieties showing only weak blackening. It may be stated, therefore, that most of the tyrosinase is found in the supernatant after extraction. The aforementioned possibility, therefore, can be excluded.

Discussion

Only a few investigations have been performed on the formation of the melanin in fishes. Fitzpatrick *et al.* (1950) demonstrated dopa oxidase activity in fish melanoma tissue. Humm and Humm (1959) made a study of the metabolism of fish melanoma. Goodrich (1933) histochemically demonstrated dopa oxidase in isolated scales of the medaka. In the tissues of normal fish, tyrosinase activity has not as yet been demonstrated. In the preceeding paper (Hishida, Tomita and Yamamoto 1961), however, the presence of tyrosinase was clearly demonstrated in isolated scales of the medaka. Furthermore, in the present study, tyrosinase was successfully extracted from adult skins of various color varieties. The enzyme does not attack monophenols, such as *p*-, *o*-, and *m*-cresols, and phenol, and diphenols such as catechol, hydroquinone, and adrenalin. Its specificity is strictly restricted to tyrosine and dopa, which is similar to that found in mammalian tyrosinase. This is very interesting from a comparative biochemical point of view, because the enzyme in amphibia shows a markedly low specificity and oxidizes most phenols to the same extent (Tomita 1960).

On the other hand, the various varieties of the medaka are distinguished by the contents of the melanin in their skin. The amount

of melanin decreases in the following order: brown variety (*BR*), orange-red and white varieties variegated with black (*B'R* and *B'r*), orange-red and white varieties (*bR* and *br*). The orange-red and the white varieties possess only the amelanotic melanophores, the variegated variety having such melanophores in the light area.

Accordingly, there arise several possibilities in the explanation of differences in the various phenotypic color manifestations from the view point of the actions of the triple allelic genes:

1) The brown variety (*B*) possesses tyrosinase, which is lacking in the orange-red and the white varieties (*b*).
2) The brown variety (*B*) possesses an adequate amount of substrate (tyrosine), whereas the orange-red and the white varieties (*b*) are deficient in it.
3) The orange-red and the white varieties (*b*) possess both enzyme and substrate, but are characterized by an inhibitory mechanism or by different metabolic pathways.
4) The orange-red and the white varieties (*b*) possess a precursor of the enzyme which, as such, is inactive, but may be activated by extraction procedures.
5) The variegated variety (*B'*) is a mosaic of the brown variety (*B*) and the orange-red or the white variety (*b*).
6) The variegated variety (*B'*) is characterized by some other mechanisms.

Skin extracts from the orange-red variety show both dopa oxidase and tyrosinase activities. The dopa oxidase activity in the orange-red variegated with black (*B'*), the orange-red, and the white varieties (*b*) is even higher than that in the brown variety (*B*), the activity of which is found to be very low. These findings rule out explanation (1). Preliminary experiments have shown that free tyrosine is present, although in very small amounts, in skins of both the brown and the orange-red varieties. This does not necessarily exclude the explanation (2), because tyrosine may be contained in some cells other than the (amelanotic) melanophores. It has further been shown that there is no difference between the water extracts of "brown" and "orange-red" skins in respect to the inhibiting action against potato tyrosinase (unpublished). The facts described above may be explained from any one of the following assumptions; i) the amelanotic melanophores in the light-colored varieties (*b*) are de-

ficient in substrate (tyrosine), ii) they contain an inactive enzyme precursor, iii) in these cells intermediate products in the process of the formation of the melanin are carried off to other metabolic pathways. It is difficult to say anything definite about the control mechanisms which play a role in the skins of various color varieties. FOSTER (1959) studied genetic control of melanogenesis in mouse skins of diverse genotypes, but he could not find any simple relationship between the natural content of the melanin and the activity of the enzyme. He concludes that genetically controlled diminished content of the melanin, as compared with an intense black condition, cannot be attributed to corresponding reduced activity of the enzyme but, rather, might be due to (1) endogenous substrate limitation or to (2) reduced efficiency of the terminal melanogenic process.

FITZPATRICK *et al.* (1959) have shown that extracts of embryonic chick and mouse eyes exhibit tyrosinase activity, in contrast to extracts of adult eyes, which are inactive. In the present study, melanin-granules obtained from the adult retinal pigment epithelium are found to lack tyrosinase activity. It will be shown elsewhere that in medaka embryos both the dopa oxidase and tyrosinase activities appear at the stage in which the melanin first accumulates in the eyes (TOMITA and HISHIDA, unpublished). These facts, therefore, are in accordance with the findings from the chick and mouse eyes. Moreover, they are also compatible with the fact that the skins containing the amelanotic melanophores (*bR* and *br*) have considerable phenol oxidase activity, while the skins containing the fully pigmented melanophores (*Br* and *ciBR*) have little activity.

The results of the present study reveal that the activities of both the dopa oxidase and tyrosinase in the adult skin are higher in the orange-red (*bR*) and the white (*br*) varieties than in the brown (*BR*) and gray (*ciBR*) varieties. This fact is quite contrary to what we at first expected. The results indicate that the activity of the phenol oxidase is very low in the melanophores in which the melanin is fully formed, suggesting that the action of gene *B* is restricted only to the stage preceeding and during the time of the formation of melanin. As the granules accompanying tyrosinase activity become more fully melanized in the later stages, perhaps tanning in a chemical sense occurs. As a result, some of the active sites on the enzyme molecules may be blocked so that apparent ac-

tivity of the enzyme is no longer noticed in the more fully melanized melanophores.

Summary

1) Tyrosinase was extracted from the skin of the medaka (*Oryzias latipes*). It oxidizes only tyrosine and dopa.

2) Tyrosinase and dopa oxidase activities in the adult skins are higher in the orange-red and the white varieties than in the brown variety, in which they are very low.

3) The differences in melanin formation among the various color varieties of the medaka are discussed.

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METHOD FOR REMOVAL OF CHORION AND FERTILIZATION OF THE NAKED EGG IN *ORYZIAS LATIPES*

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Since the teleostean egg is enclosed in the chorion which spermatozoa cannot penetrate except the region of the micropyle, polyspermic fertilization never occurs even if the egg is inseminated by dense sperm suspension. As a matter of fact, once the chorion is removed, both herring and salmon eggs receive more than one spermatozoon (YANAGIMACHI, 1957; YAMAMOTO, T. S., 1958; KANOH and T. S. YAMAMOTO, 1957). In the Medaka, *Oryzias latipes*, the present author found a way to remove the chorion and also succeeded in monospermic fertilization of the naked eggs. The naked embryos can be kept alive for various lengths of time in sterilized medium. These results will be presented in the following pages.

METHODS

YANAGIMACHI (1957) succeeded in removing the chorion from the unfertilized herring egg by mechanical means. KANOH and T. S. YAMAMOTO (1957) and T. S. YAMAMOTO (1958) removed the chorions of the unfertilized eggs of both salmon and herring by a dual treatment with acidulated and pancreatin-containing Ringer. On the other hand, in *Oryzias latipes*, ISHIDA (1944 a, b, 1948) reported that the use of pancreatin or trypsin singly dissolves the chorions if left in the solution for more than 20 hours, although he casually mentioned the ultimate dissolution of the chorion by the natural hatching enzyme too.

As far as the author's experience goes, the methods reported in the first three papers are not effective for *Oryzias* eggs and ISHIDA's method requires too long a treatment to obtain viable eggs. However, it was noticed that a mixture of the natural hatching enzyme and pancreatin could attack the chorion without affecting the development

of the embryos.

1) Preparation of the hatching enzyme

The hatching enzyme is prepared from about 30 embryos of the age of one day before hatching. After disinfecting with 30% alcohol and removing the yolk and chorion, the 30 embryos proper are crushed with a small glass homogenizer in cold in 0.05 ml. of distilled water and 0.025 ml. of phosphate buffer of pH 8.0 (ISHIDA, 1944 a). The homogenate is centrifuged at 2000-3000 g for 10 minutes in cold, and the supernatant is employed as a solution of the hatching enzyme.

Since the speed of dissolution of the chorion depends on the activity of the hatching enzyme used, embryos in the best condition should be selected as the enzyme source.

2) Enzyme mixtures tested and their efficacies

The following three kinds of mixtures are tested.

a) 0.05 ml. of the hatching enzyme solution+0.05 ml. of redistilled water or 0.05 ml. of the Ringer solution.*

b) 0.05 ml. of the hatching enzyme solution+0.05 ml. of 10% trypsin solution.**

c) 0.05 ml. of the hatching enzyme solution+0.05 ml. of 3% pancreatin solution.***

About 30 embryos are tested for each solution to compare the efficiencies of dissolution of the chorion. The rate of dissolution is greater in the order of from a) to c) which holds equally for unfertilized and fertilized eggs.

3) The procedure for the removal of the chorion

Five eggs are selected and slits are made in the chorions with a sharp knife in the Ringer solution to secure faster access of the enzyme to the interior of the chorion. They are then transferred to about 0.1 ml. of the enzyme mixture and incubated at 30° C. When

* M/7.5 NaCl 100 parts + M/7.5 KCl 2 parts + M/11 $CaCl_2$ 2.1 parts, pH adjusted to 7.3 by N/10 $NaHCO_3$ (YAMAMOTO, T., 1939 a, 1944 a).

** WAKO's product.

*** KONISHI's, MIKUNI's and HOEI's commercial products. 5% solution sometimes activates the eggs or makes them shrink, while 3% solution scarecely has such an effect. The KONISHI's product is found to be more preferable.

the chorions become soft enough, the eggs are put back to the Ringer and the chorions are removed mechanically with forceps.

The naked eggs thus obtained are cultured in 1/2 or 1 strength of the Ringer, which have previously been sterilized by an autoclave at 2× atmospheric pressure for 30 minutes.

RESULTS

Part I. Removal of the chorion and its effect on development

1) Process of the dissolution of the chorion

The chorion of *Oryzias* egg consists of an outer heterogeneous layer and an inner homogeneous layer (ISHIDA, 1944 a). The villi and attaching filaments are present on the former.

i) Unfertilized egg. In the unfertilized egg, because of the close application of the chorion to the egg surface (Plate 4, Fig. 1), the mechanical removal of the chorion is impossible in spite of the fact that the chorion has not been hardened.

On treating the unfertilized egg with the hatching enzyme-pancreatin mixture, as the first step, small holes are made to appear in the outer layer in 15-20 minutes as in ISHIDA's description (1944 a, b). As the second step, the inner layer begins to be dissolved and the chorion separates from the egg surface. As the chorion becomes thinner, it elevates itself further from the egg surface to form a space comparable to the perivitelline space of the fertilized egg (Fig. 2). When this stage is reached, the chorion can be stripped with forceps in the Ringer solution.

Since too long an exposure to the enzyme mixture results in activation or shrinkage of the egg, it must be returned to the Ringer solution soon after the chorion separates from the egg surface.

ii) Fertilized egg. In the fertilized egg kept in the hatching enzyme-pancreatin mixture, small holes are noticed in the outer layer which increase in number but enlarge only slightly after 15-20 minutes. The inner layer slightly swells first and gets thinner and disappears in 30-60 minutes. These changes in the inner layer spread from the edge of the slit toward other parts. Since the hardening of the chorion following fertilization is due to the hardening of the inner layer alone (NAKANO, 1956), the remaining outer layer can easily be eliminated with forceps (Table 1).

Table 1. Time course of dissolution of the chorion in the fertilized egg

Exposure time in the hatching enzyme-pancreatin mixture	15–20 min.	30–40 min.	40–60 min.	60–120 min.
Outer layer	Slightly perforated	Perforated in places	Perforated in places	Considerably perforated
Inner layer	Slightly dissolved	Fairly dissolved	Almost dissolved	Completely dissolved
Means of removal of the chorion	By forceps with difficulty	By forceps with care	By forceps with ease	Gentle blowing by pipette

2) *The rate of success of the operation*

Since unfertilized eggs are liable to be wounded or activated during the operation, it is rather difficult to obtain intact unactivated naked eggs (Fig. 3) and the rate of success is at best 60–70%.

Concerning eggs after fertilization, although the mode of dissolution remains the same through various developmental stages, since yolk region of the embryo is more susceptible to mechanical injury, there is a difference in the rate of success between before and after epiboly; that is, respectively 70–80% and nearly 100%.

3) *Later development of the naked egg*

i) *Unfertilized egg.* If naked unfertilized eggs are inseminated with a sperm suspension of the usual density (about 1/2000), fertilization is polyspermic. On the other hand, extremely dilute sperm can induce monospermic fertilization if the sperm is delivered to the egg surface by a capillary. The use of a capillary is to prevent the spreading of the spermatozoa and also to keep down the number of spermatozoa which arrive at the egg surface simultaneously.

When cultured in non-sterilized medium, most of the monospermic naked eggs die at the morula or gastrula stage (Fig. 5). However, if the medium is sterilized, the best examples develop up to the heart beating stage (Fig. 7). Accordingly, it can be said that the chorion is protecting the egg from polyspermy and from mechanical disturbance as well as from attack of bacteria.

ii) *Fertilized egg.* In non-sterilized medium, fertilized eggs denuded at the early cleavage stage can reach the stage of embryo formation (Fig. 6), but those denuded at the heart beating stage can

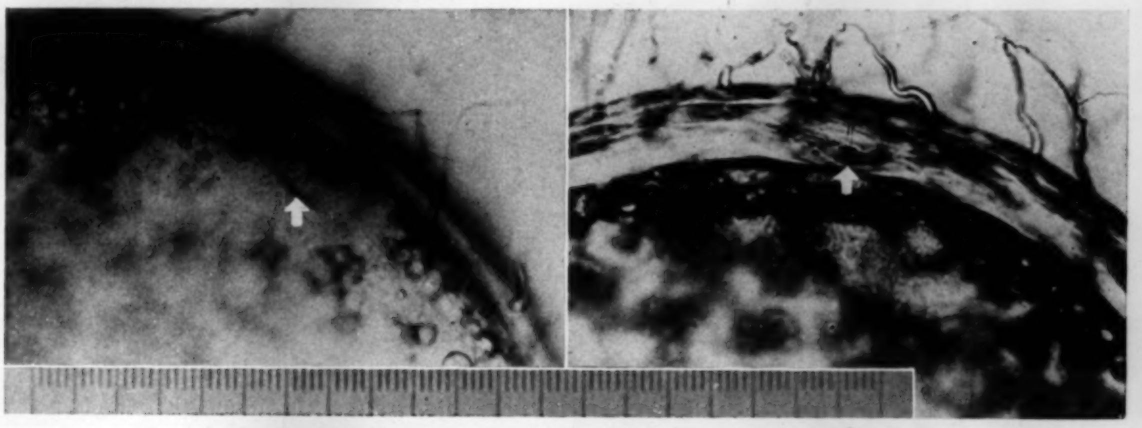
develop to the swimming stage (Fig. 8). On the other hand, the best example of naked eggs kept in sterilized medium from the 2-cell stage (Fig. 4) succeeded in reaching the swimming stage.

Part II. Fertilization of the naked egg

Before proceeding to the description of the fertilization of denuded eggs, some remarks concerning normal fertilization will be given which have a close bearing to the future discussion.

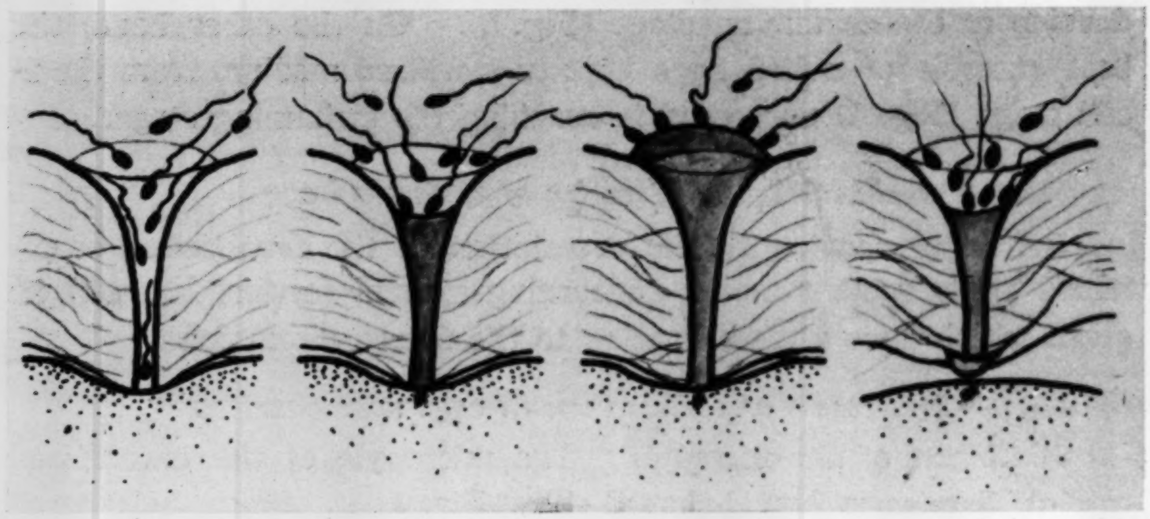
1) Process of fertilization in eggs with chorions

i) Closure of the micropyle. The micropyle of the unfertilized egg of *Oryzias* is funnel-shaped (Text-Fig. 1 a). Since the tapered end of the funnel is as narrow as the head of a spermatozoon, when several spermatozoa have entered the funnel, they line up in a single row. When the first spermatozoon reaches the egg surface and the cortical alveoli begin to breakdown, spermatozoa following the first one are pushed out toward the entrance of the micropyle by colloidal substance which comes out from the inside through the narrow end of the funnel (Text-Fig. 2 a, b). Occasionally, this substance reaches the uppermost part of the funnel or even bulges out (Text-Fig. 2 c). Such a dome formed over the micropyle was known in *Stiroztedion vitreum* as early as in 1890 (REIGHARD) and it seems to correspond to the structure which K. YAMAMOTO (1952) called the 'plug' in the micropyle of *Limanda shrenki.* The same situation holds in the eggs of the salmon, herring (KANOH, 1957; YANAGIMACHI and KANOH, 1953) and sturgeon (GINSBURG, 1959).



a *b*

Text-Fig. 1. Structure of the micropyle. *ca.* ×82.
a. Micropyle of unfertilized egg. *b.* Micropyle of fertilized egg. 1-2 minutes after fertilization with the cup-like structure.



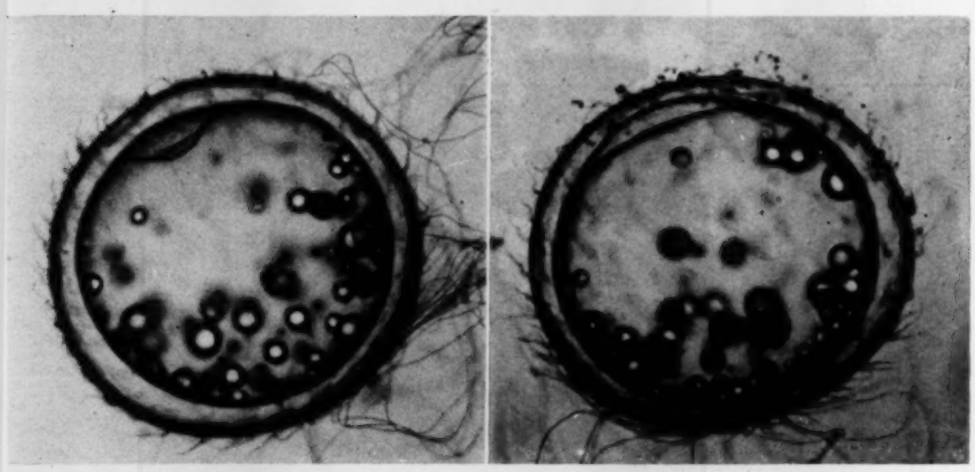
a b c d

Text-Fig. 2. Process of the closure of the micropyle in *Oryzias latipes*. The explanations are given in the text.

Whithin a few minutes, a new cup-like structure is formed at the bottom of the funnel as if the micropyle were completely closed from within (Text-Figs. 1 b and 2 d). This structure seems to be quite specific to the eggs of *Oryzias*.

The encounter of the two pronuclei immediately follows, since the egg pronuleus is located in the superficial protoplasmic layer directly under the micropyle.

ii) *Formation of the blastodisc.* The breakdown of the cortical alveoli and the separation of the chorion from the egg surface follow



a b

Text-Fig. 3. Blastodisc formation of the intact egg. *ca.* ×33. *a*. Early lens-shaped blastodisc, 40 minutes after fertilization. *b*. Completed blastodisc, 1 hour after fertilization.

the sperm entry (YAMAMOTO, T., 1939 a, b, 1943 a, 1944 a, b). When the alveolar breakdown is completed (in 2–3 minutes), the bipolar differentiation follows (YAMAMOTO, T., 1958).

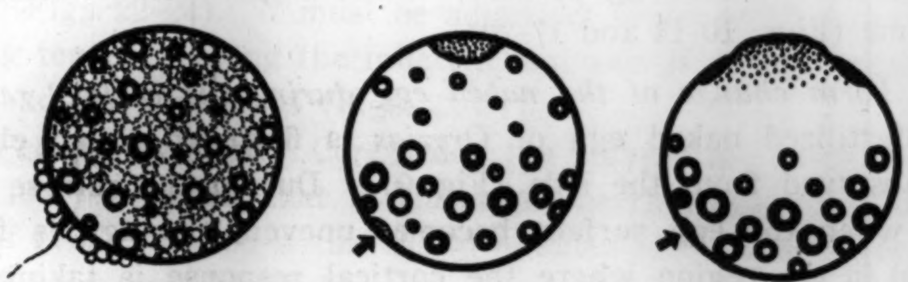
In the process, the protoplasm accumulates at the animal pole so that the protoplasmic layer takes a lens-shape in the optical section during early stage (Text-Fig. 3 a). However, as more protoplasm accumulates, the layer subsequently bulges out on the surface to become the typical blastodisc (Text-Fig. 3 b).

2) *Fertilization of the naked egg*

i) Sperm entry. In the case of the naked egg, it is a question whether the sperm still penetrates only into the animal pole region regardless of the absence of the chorion. To test this point, extremely dilute sperm is brought to the egg surface through a capillary at various parts of the egg surface other than the animal pole. It is found that the spermatozoon can enter at any part of the egg, initiating the alveolar breakdown from there (Text-Fig. 4 a).

In this case, a lens-shaped structure is formed at the sperm entrance area but this lens is extremely small and it scarcely bulges out on the surface and eventually disappears (Text-Fig. 4 b, c).

In whichever part of the egg a spermatozoon may enter, the real blastodisc is formed at the animal pole as the result of the bipolar differentiation (Text-Fig. 4 c) and normal development ensues. Consequently, the spermatozoon must migrate a longer distance to the egg pronucleus than in the normal case causing a delay of the first cleavage.



Text-Fig. 4. Diagramatic illustration of the formation of the blastodisc at the animal pole irrespective of the position of the sperm entrance in monospermy of the naked egg.

ii) Release of colloidal substance during the cortical change. Under the intact chorion, when the cortical alveoli breakdown, their contents

are ejected from the egg surface and are eventually deposited on the inner surface of the chorion; this, in turn, causes hardening of the chorion (NAKANO, 1956; OHTSUKA, 1957, 1960). However, since it is rather difficult to see this process under the chorion, T. YAMAMOTO (1951, 1958) resorted to vital staining of the alveolar contents with Janus green B or neutral red. In the naked egg, on the contrary, the process of the ejection of the colloidal content of the alveoli can be followed with ease without staining (Plate 5, Figs. 9-16).

At the very beginning of the breakdown, the outline of the alveoli becomes indistinct and the content is slowly pushed out of the egg surface, somewhat like the case of the cortical granules of sea urchin eggs as reported by ENDO (1952). Ejected masses soon swell to transparent spheres on the egg surface (Figs. 10-15). But within 15-20 minutes following insemination or activation, the spheres detach themselves and fall to the bottom of the container and finally dissolve away (Figs. 13-16. See the darkened substratum). Comparing with T. YAMAMOTO's (1958) description for intact fertilized eggs, disappearance of the ejected spheres of the naked egg seems to take place much slower. Whether this is due to a real difference in the speed of disappearance or to a difference in the length of period during which the visibility lasts must be determined in future. In the lamprey, however, spheres are seen attached to the egg surface for a longer period (KUSA, unpublished).

During the release of the colloid, the egg surface of *Oryzias* temporarily becomes uneven, the contour of individual oil droplets standing out on the egg surface, which regains smoothness in a short time (Figs. 10-14 and 17-20).

iii) *Form change of the naked egg during the cortical response.* The unfertilized naked egg of *Oryzias* is flattened to an ellipsoid when observed from the side (Fig. 9). During the release of the colloid when the egg surface becomes uneven, the egg is further flattened in the region where the cortical response is taking place (Figs. 10-11). After the cortical response is over, the egg bulges higher than before fertilization, recovering the smoothness of the egg surface (Figs. 12-16). After a while, the egg sometimes flattens quite spontaneously once again which is followed by a recovery.

For the sake of a comparison, the value of 'the tension at the surface' in *Oryzias* eggs is calculated by the sessile drop method

(HARVEY and FANKHAUSER, 1933; HARVEY and DANIELLI, 1938). The densities of the Ringer and the naked unfertilized egg are found to be 1.008 and 1.047 respectively. The value is 0.18 dyne/cm. in the unfertilized egg (Fig. 9) and is 0.07 dyne/cm. in the most flattened condition (Fig. 13). Although the above two values match the figure of *Triturus viridescens* (HARVEY and FANKHAUSER, 1933) in the order of magnitude, when the *Oryzias* egg bulges later (Fig. 15), the tension at the surface rises as high as 1.34 dyne/cm. The first flattening may be the result of the alveolar breakdown, but there is, at present, no explanation for the second spontaneous flattening.

iv) *Polyspermy.* If the naked egg of *Oryzias* is inseminated with the sperm suspension of customary density (*ca.* 1/2000), the fertilization is usually polyspermic. In this case, small lens-shaped protoplasmic accumulations are formed, each corresponding to the entry of a spermatozoon besides the main blastodisc at the animal pole (Plate 6, Figs. 21–22). This is further confirmed by sections* (Figs. 25–28). On the contrary, in the polyspermic herring egg, it is said that only one blastodisc is formed at the normal position (YANAGIMACHI, 1957). When more than one protoplasmic accumulations are located close by in *Oryzias*, they often fuse. At any event, the main blastodisc always has capacity for division (Figs. 23–24), while, protoplasmic accumulations sometimes cleave abnormally or do not cleave at all.

Since oil droplets tend to move away from the accumulation, when many of them are formed scattered randomly over the egg surface, final distribution of the oil droplets becomes irregular or rather chaotic (Figs. 22–24). It must be admitted, however, that the differentiating tendency along the inherent egg axis is stronger than those along abnormal directions (Figs. 23–24).

v) *Polyspermy block.* Concerning the polyspermy block, YANAGIMACHI (1957) reported that in the naked herring egg, refertilization occurs if reinseminated within 20–30 minutes after fertilization.

In *Oryzias* eggs which have been inseminated or activated previously, spermatozoa can still enter into the egg as long as the second

* For fixation, P.F.A. (Picric acid sat. aq. sol. 75: formol 15: Acetic acid, *glacial* 10: Urea 1) or F.A.A. (Formol 6: Alcohol 16: Acetic acid 1, plus 30 parts water) fixative was used and the preparations were sectioned by the paraffin method at 10 μ and stained with HAIDENHEIN's Hematoxylin.

insemination is given within about 20 minutes. However, by the time when the bipolar differentiation advances (more than 60 minutes after fertilization or activation) spermatozoa become no longer able to enter into the egg. This fact indicates that the polyspermy block on the naked egg of *Oryzias* does not take effect immediately after fertilization like in sea urchin eggs (ISHIDA and NAKANO, 1947, 1950; SUGIYAMA, 1947, 1951, etc.).

Although KANOH (1957) considered such a slow change which requires more than 20 minutes could be regarded as a mechanism of polyspermy block, monospermic fertilization of fish eggs is insured by the micropyle which closes after admitting only one spermatozoon.

SUMMARY

1) The method to obtain the unimpaired naked egg of *Oryzias latipes* is as follows:

a) The egg, the chorion of which has previously been slit is exposed to the mixture of the hatching enzyme and pancreatin to dissolve the inner layer of the chorion.

b) The removal of the remaining outer layer of the chorion is done with forceps in the Ringer solution.

2) Spermatozoa can enter at any part of the naked egg and usually cause polyspermy, although in a certain experimental condition monospermic fertilization is not impossible.

3) The early developmental changes of the naked egg are described.

4) The naked monospermic egg develops normally in 1/2 or 1 strength of the sterilized Ringer solution.

The author particularly wishes to express her gratitude to Mr. M. SAWA who was the first to find the efficacy of this method. Her thanks are also due to Prof. K. DAN for his invaluable advice and kind help in the preparation of the manuscript.

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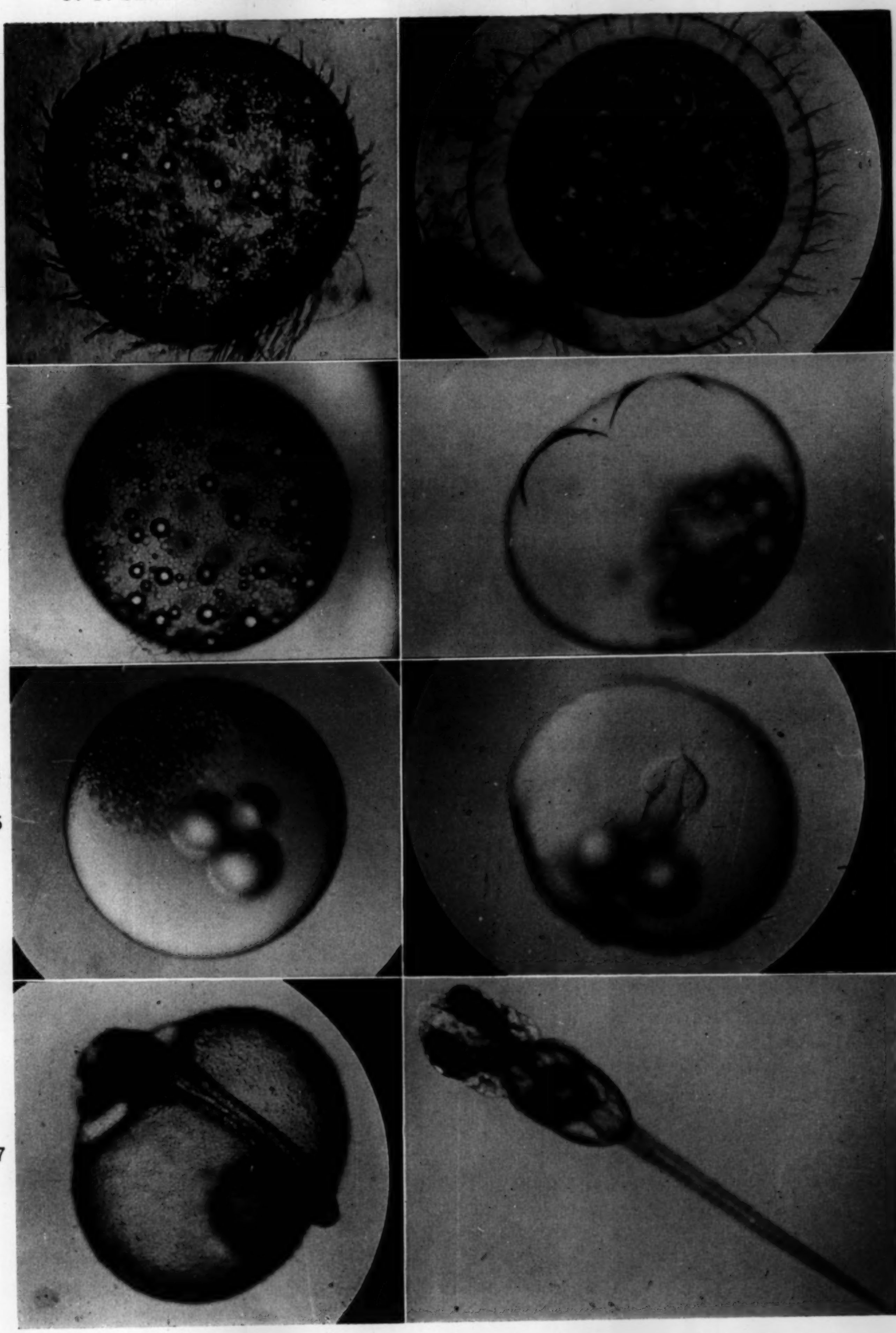
(*Manuscript received: 29. Sep., 1960*)

EXPLANATIONS OF PLATE 4

Removal of the chorion and development of the naked egg. *ca.* ×33.

Fig. 1. Intact unfertilized egg.
Fig. 2. Unfertilized egg in a mixture of hatching enzyme and pancreatin. Thin chorion has risen.
Fig. 3. Unfertilized egg deprived of the chorion. Note the presence of the cortical alveoli.
Fig. 4. 2-cell stage.
Fig. 5. Morula stage.
Fig. 6. Stage of embryo formation.
Fig. 7. Heart beating stage.
Fig. 8. Swimming stage.



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Explanations of Plate 5

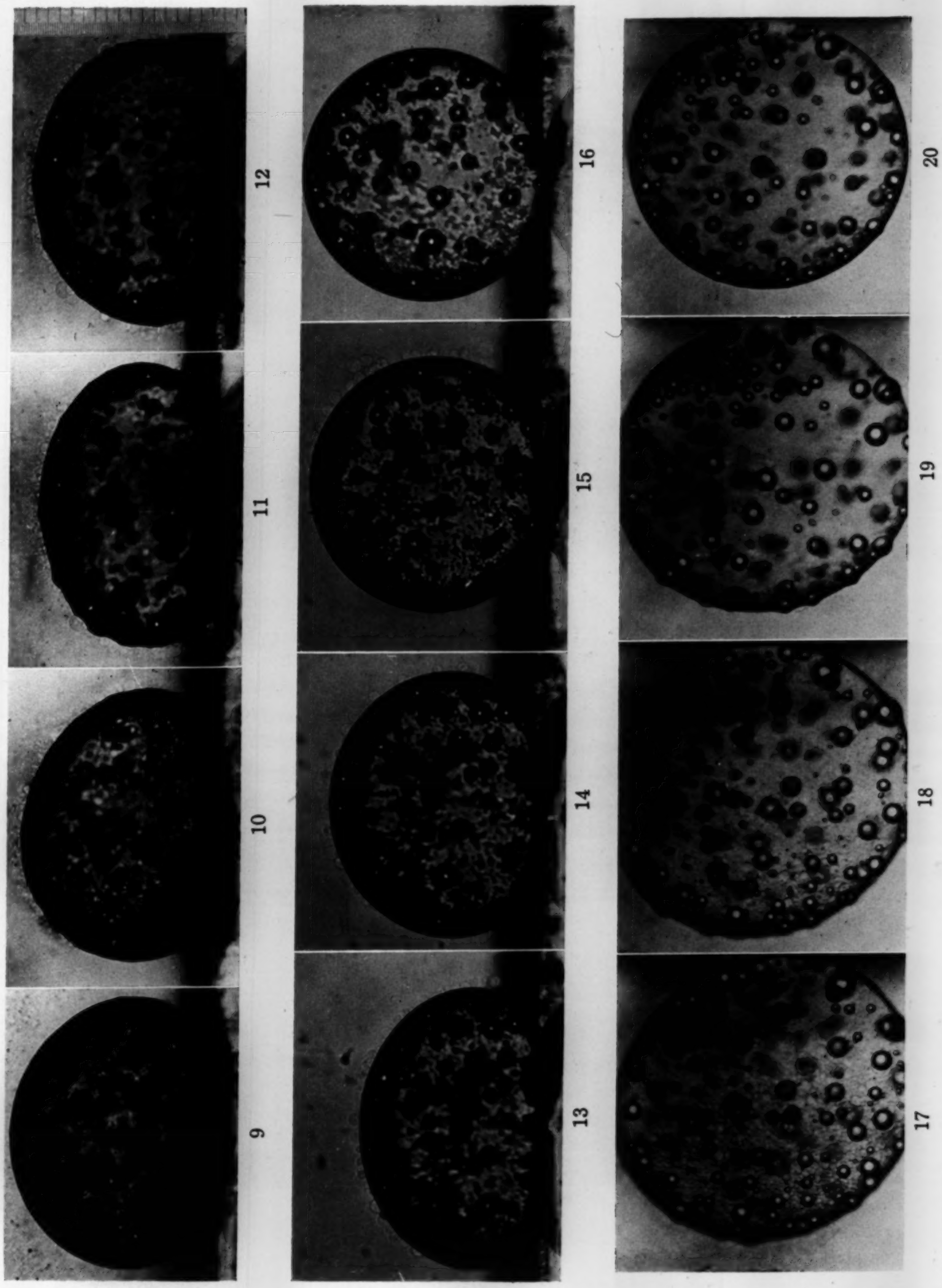
Cortical change by fertilization or activation in the naked egg.

Release of the content of the cortical alveoli and an accompanying flattening of the egg and its later recovery. (*Side view*), *ca.* $\times 33$.

Fig. 9. Naked unfertilized egg. (The same egg as in Fig. 3)
Fig. 10. 1 minutes after insemination.
Fig. 11. 1.5 minutes.
Fig. 12. 2.5 minutes.
Fig. 13. 3.5 minutes.
Fig. 14. 6 minutes.
Fig. 15. 10 minutes.
Fig. 16. 20 minutes.

The wave of breakdown of cortical alveoli.

Fig. 17. 1 minutes after activation.
Fig. 18. At 1.5 minutes.
Fig. 19. At 2 minutes.
Fig. 20. At 5 minutes.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Explanations of Plate 6

Polyspermic development.

Ooplasmic polarlization in the polyspermic egg. ca. $\times 33$.

Fig. 21. Lens-shaped accumulations of protoplasm.

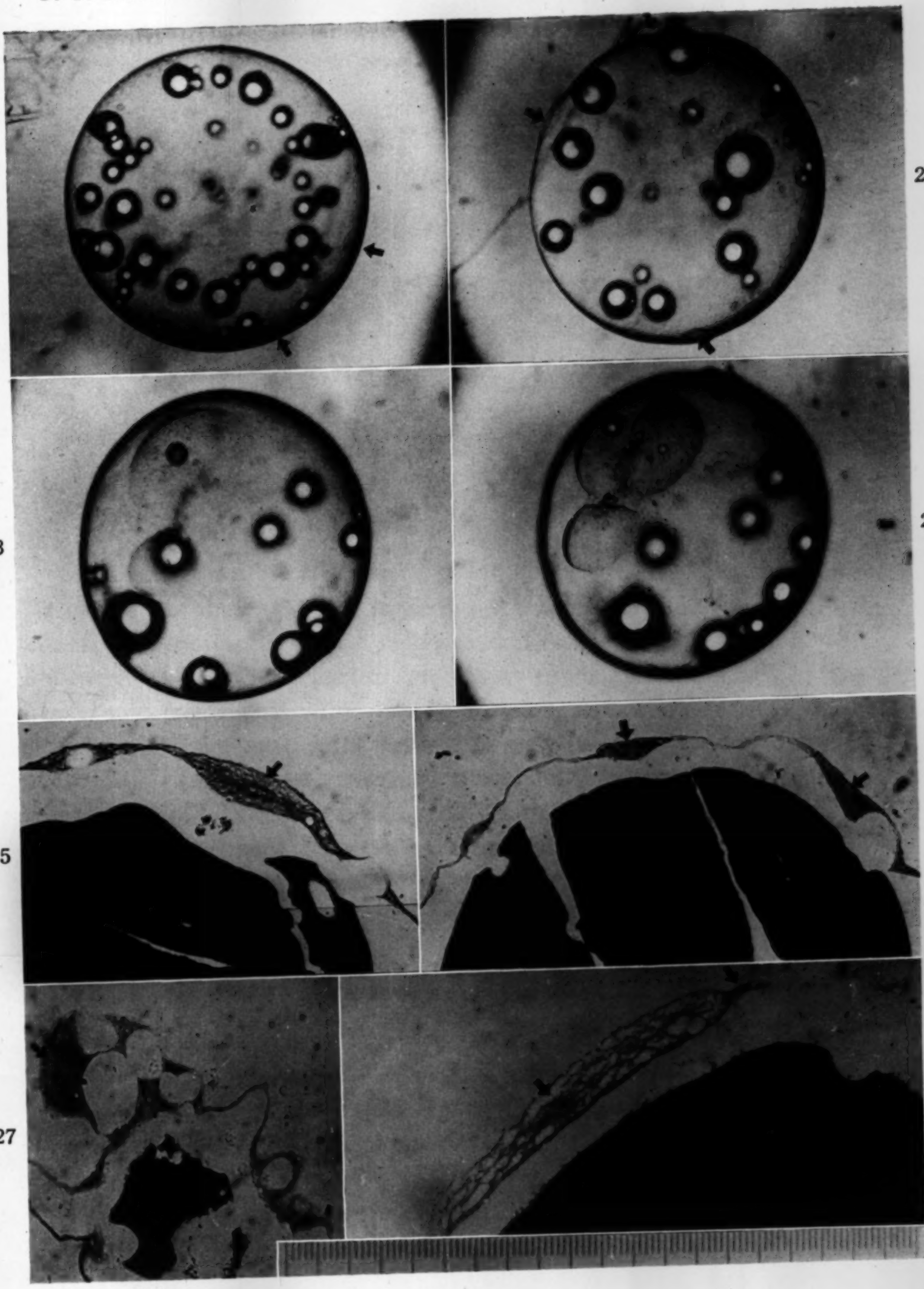
Fig. 22. Protoplasmic accumulations bulging.

Figs. 23, 24. Abnormal cleavage of main blastodisc at the animal pole and chaotic distribution of oil droplets.

Sections of polyspermic egg. ca. $\times 82$.

Figs. 25, 26, 27. Serial sections of protoplasmic accumulations fixed by P.F.A. The nuclei are present in each accumulation.

Fig. 28. Section of the protoplasmic accumulation fixed by F.A.A. Mitotic figures are seen.



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EMBRYOLOGIA, Vol. 5, No. 4, pp. 369–375 (March, 1961)

CYCLIC CHANGES OF THE CORTEX AND THE CYTOPLASM OF THE FERTILIZED AND THE ACTIVATED SEA URCHIN EGG

III. SUSCEPTIBILITY OF ARTIFICIALLY ACTIVATED EGGS TO CLEAVAGE-INDUCING ACTION OF HYPERTONIC, DNP- AND NaN_3-SEA WATER*

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In LOEB's "double treatment" method for artificial parthenogenesis of sea urchin eggs, the hypertonic treatment is usually applied about 10–20 minutes after membrane formation (cf. LOEB, 1913). Recently, the author carried out some experiments to test if the hypertonic treatment is effective even when it is applied at a later stage and if the susceptibility of the egg to hypertonicity changes cyclically. In another series of experiments, it was further studied whether or not DNP and NaN_3 could be used as a second agent in the double treatment for the induction of cleavage. Some results obtained in the above-mentioned experiments are reported in the present paper.

MATERIAL AND METHODS

Eggs of the following four species of sea urchins were used as material: *Pseudocentrotus depressus, Hemicentrotus pulcherrimus, Temnopleurus toreumaticus* and *Anthocidaris crassispina.* Since all of them gave essentially the same results, the data of the first two species alone will be described.

As activating agents were employed butyric acid-sea water (6 cc. N/10 butyric acid + 50 cc. sea water), urea solution (1 M) and thymol solution (30 cc. saturated thymol solution + 70 cc. sea water).

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* A part of this work was preliminarily reported in Japanese (KOJIMA, 1958).

Hypertonic sea water was prepared by adding 8 cc. of 2.5 N NaCl solution to 50 cc. of sea water.

M/500 DNP in sea water and M/10 NaN_3 in distilled water were taken as stock solutions, which were diluted to appropriate concentrations with sea water according to the purpose of experiments.

Experimental Results

1) *Hypertonic treatment*

In *Hemicentrotus* eggs, the hypertonic treatment is effective for the induction of parthenogenetic development when it is applied 10–20 minutes after the artificial membrane formation, whereas it is ineffective if applied 30 minutes after the membrane formation. On the other hand, cyclic growth of the monaster at regular intervals (about 50–60 minutes in *Hemicentrotus* eggs) has been reported in the eggs activated by butyric acid, urea or thymol (Kojima 1960). This suggests that stages in which hypertonic treatment is effective may be occurring cyclically in accordance with the cytoplasmic change following membrane formation. Therefore, this possibility was tested.

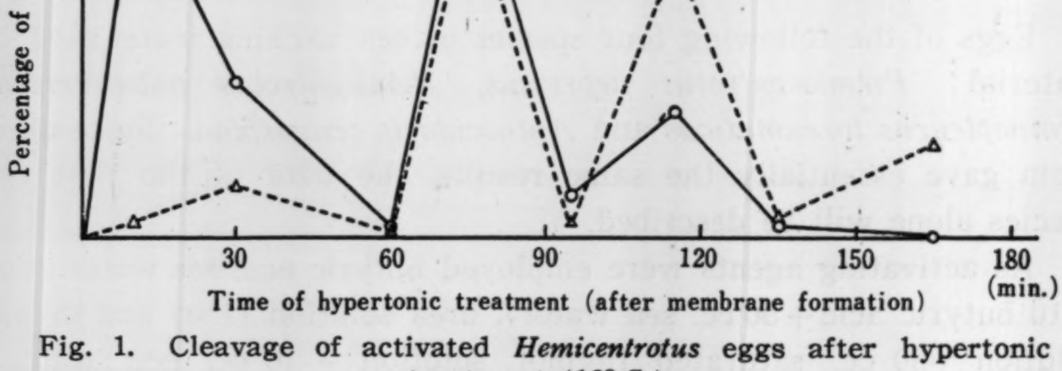

Fig. 1. Cleavage of activated *Hemicentrotus* eggs after hypertonic treatment (16° C.).

Eggs were exposed to hypertonic sea water for 40 minutes (–○–) or 50 minutes (–△–). The figures in the upper part show the cyclic appearance of the monaster in an activated egg which has not been treated with hypertonic sea water.

It has become clear that peaks of the effectiveness of the hypertonic treatment occur not only at 10–20 minutes after activation but also at 60–70 minutes, that is, at the early monaster stage and even at the early stages of the second and third monasters. Figure 1 illustrates these experiments.

2) *DNP- and NaN_3-treatment*

Eggs which have been activated by butyric acid, urea or thymol are immersed in DNP-sea water (M/500–M/4,000) or NaN_3-sea water (M/100–M/1,600) for 2 hours.

As shown in Table 1, when eggs are put into NaN_3-sea water 10–30 minutes after activation, cleavage generally fails to take place, but if the same treatment is applied 60–70 minutes after activation, cleavage is induced in a fairly high percentage. Such a high percentage, however, is not obtained when eggs are treated 80–90 minutes after activation. In general, the eggs which have once started to cleave develop into swimming blastulae (Fig. 2).

Table 1. The percentage of cleavage in *Hemicentrotus* eggs activated by butyric acid, followed by 2 hours' NaN_3-treatment (17° C.)

Concentrations of NaN_3 ↓ / Time when eggs were introduced into NaN_3-sea water (minutes after fertilization) →	10	20	30	40	50	60	70	80	90
M/100	0	0	1	2	8	44	51	10	12
M/200	0	0	1	0	11	39	37	9	9
M/400	0	0	0	2	19	45	35	4	11
M/800	0	0	1	3	16	44	15	5	9
M/1,600	0	0	0	1	25	27	6	7	10

Effect of DNP on activated eggs is almost the same as that of NaN_3 (Fig. 3). However, eggs treated with DNP do not develop beyond the 8-cell stage. DNP as well as NaN_3 is most effective when the treatment is applied at the early monaster stage.

It is interesting to note that such a cleavage-inducing effect of both DNP and NaN_3 is observed not only at the first monaster stage but also at the second one. In Figure 4 is shown one of the results of experiments with DNP.

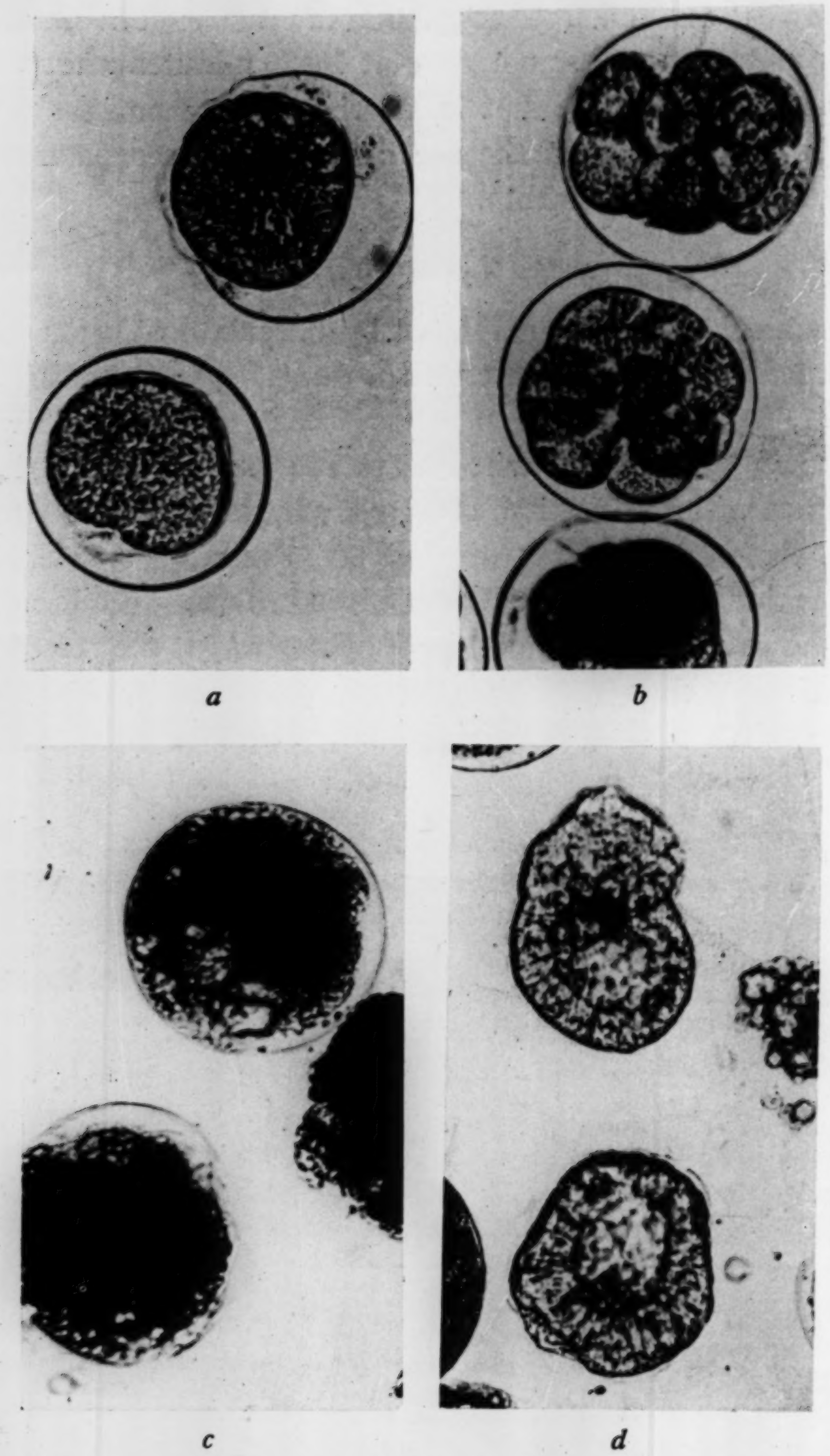

Fig. 2. Cleavage of activated *Hemicentrotus* eggs after NaN_3-treatment for 2 hours (17° C.).
a and *c*: eggs treated with NaN_3 30 minutes after activation. *b* and *d*: eggs treated with NaN_3 60 minutes after activation.
(*a*) and (*b*) were photographed 5 hours, and (*c*) and (*d*) 24 hours after activation.

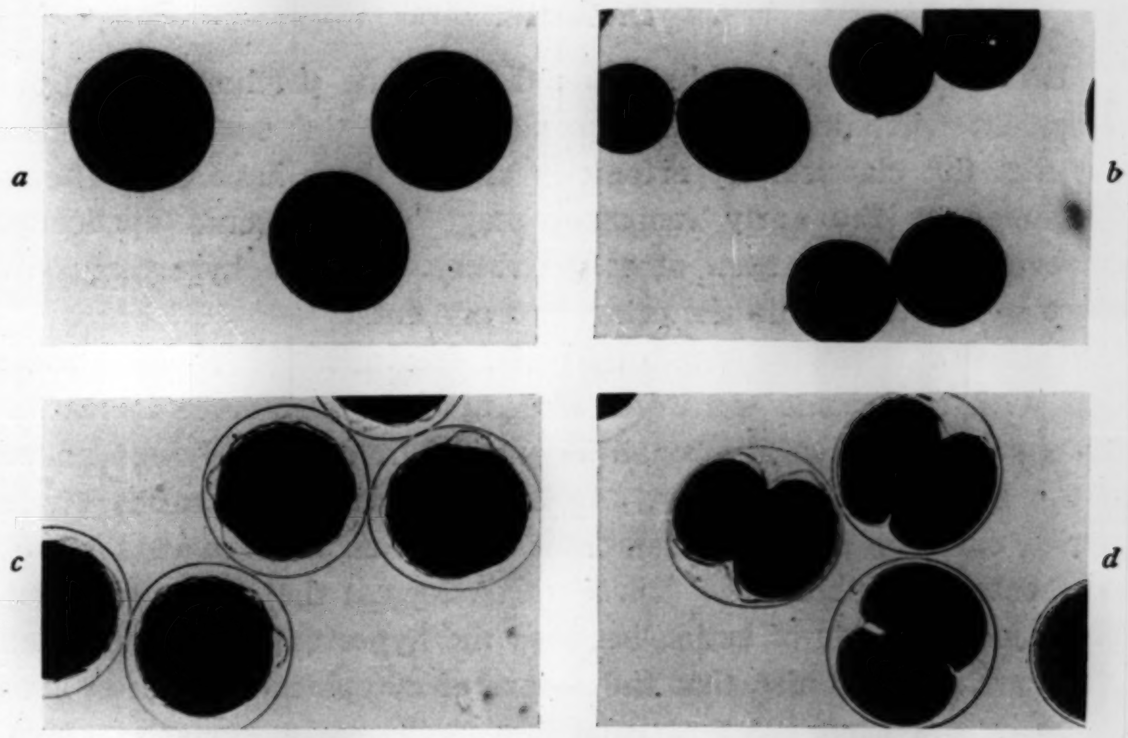

Fig. 3. Cleavage of activated ***Pseudocentrotus*** eggs after DNP-treatment for 2 hours (17° C.).

a and *c*: eggs treated with DNP 30 minutes after activation. *b* and *d*: eggs treated with DNP 90 minutes after activation.

Eggs shown in (*a*) and (*b*) were activated with 1 M urea solution; those in (*c*) and (*d*) with 6% butyric acid sea water.

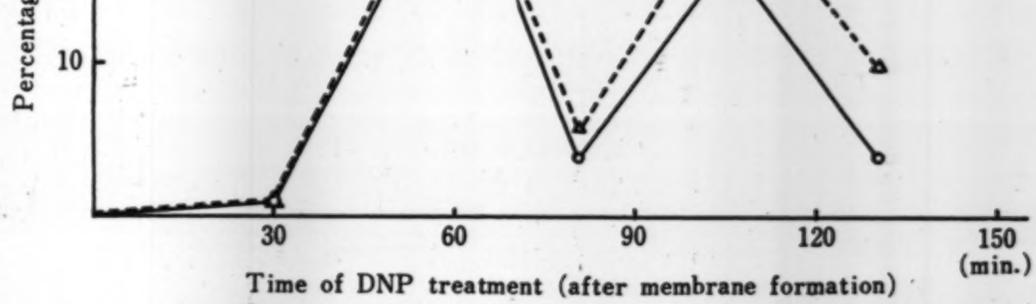

Fig. 4. Cleavage of activated ***Hemicentrotus*** eggs after DNP-treatment (16° C.).

Eggs were exposed to M/2,000 (-○-) or M/4,000 (-△-) DNP-sea water for 2 hours. The figures in the upper part show the cyclic appearance of the monaster in an activated egg left untreated with DNP-sea water.

Discussion

DNP and NaN_3 have been found to induce parthenogenetic development when used as the second step of the double-treatment just like the classical hypertonicity and all these have their effects if applied at the early monaster stage which recurs cyclically. However, if applied soon after activation only the hypertonic sea water is effective while the other two are not.

Ishikaka (1957) reported that the treatment of *Hemicentrotus* eggs with hypertonic sea water after artificial membrane formation was made ineffective, if a small amount of DNP or NaN_3 was added to the hypertonic sea water. In his case, since the treatment was carried out soon after the membrane formation, the reagents might have acted as inhibitors. These facts suggest that there are some differences in mode of action between the hypertonic sea water and DNP or NaN_3, and also that the nature of cytoplasm soon after activation is somewhat different from that at the early monaster stage. Further investigations are necessary to elucidate this point.

Summary

1. The hypertonic treatment is effective for parthenogenesis of sea urchin eggs not only when it is given soon after membrane formation but also when given at the early part of the monaster stages, which appear cyclically after membrane formation.

2. When activated eggs are exposed to DNP-sea water (M/500–M/4,000) or NaN_3-sea water (M/100–M/1,600) at the early monaster stage for 2 hours, the eggs start to divide in fairly high percentages, and, in the cases of NaN_3, they develop into swimming blastulae. The susceptibility of activated eggs to DNP and NaN_3 changes cyclically and a receptive period occurs at each monaster stage.

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EMBRYOLOGIA, Vol. 5, No. 4, pp. 376–383 (March, 1961)

SOME QUANTITATIVE ASPECTS OF THE ACROSOMAL REACTION TO JELLY SUBSTANCE IN THE SEA URCHIN

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Although it has been known for some time from morphological evidence (DAN, 1952; AFZELIUS and MURRAY, 1957) that the acrosome of the sea urchin spermatozoon reacts to the presence of the jelly substance of its own species by forming a delicate process[1], the only quantitative evidence bearing on this subject to be presented so far is that of COLLIER (1959). Working with *Strongylocentrotus purpuratus*, he found that about 60% of the spermatozoa suspended in a solution of jelly substance ("homologous fertilizin") underwent such a reaction, in contrast to about 10% of those suspended in sea water. His results also show that the percentages of reacting spermatozoa tend to increase as the concentration of fertilizin rises.

Preliminary experiments performed on *Hemicentrotus* gametes in collaboration with E. NAKANO, and on gametes of *Lytechinus variegatus*, with C. B. METZ at the Alligator Harbor Laboratory of Florida State University, indicate similar trends in these species. The experiments to be reported here are a continuation of that work; while they more or less repeat those of COLLIER, the authors feel that the confirmation of his results with different material sufficiently justifies their publication.

MATERIAL AND METHODS

The sea urchins used were the regular urchins *Pseudocentrotus*

[1] Referred to in earlier papers as: "a central core or fiber of some sort" (DAN, 1952, p. 58), "highly pliable strand" (*loc. cit.*, p. 60), "acrosome process" (DAN, 1954, p. 337), "acrosome filament" (*loc. cit.*, p. 341), "flexible strand" (DAN, 1956, p. 366), "Filament" (*loc. cit.*, p. 370), "sturdy clump of apparently fibrous material" (*loc. cit.*, p. 370), "filament—for want of a more precise term" (DAN, 1960, p. 13).

depressus and *Hemicentrotus pulcherrimus.* "Dry sperm" was secured by isotonic KCl-induced shedding in the case of *Pseudocentrotus*, and from excised *Hemicentrotus* testes. In each experiment the dry sperm from three animals was mixed to reduce individual variation; part of this stock was used immediately after collection and the rest stored in a refrigerator at about 10° C. for 24 hours.

Solutions of the egg jelly were prepared either by acid sea water extraction at pH 5.5 or by simply removing and filtering the supernatant from a 50% suspension of unfertilized eggs which had stood with occasional stirring for about an hour. The concentration of jelly substance in such solutions was bioassayed by the usual method of testing their capacity to agglutinate freshly shed spermatozoa. In order to standardize the titers as much as possible, the dilution series were made on a glass spot plate, equal amounts of a fresh 1% sperm suspension were added to one dilution at a time, and the mixture observed immediately at 100 × magnification for signs of agglutination. The titer was fixed at the highest dilution in which definite clumping could be seen to last for at least 15 seconds.

Since the difference between unreacted and reacted sea urchin spermatozoa cannot be established with sufficient accuracy at light microscopic magnifications, it is necessary to use electron microscopy for making counts to determine the percentages of reacted acrosomes in the test jelly solutions of various concentrations. In the experiments, serial dilutions of jelly substance in sea water were added in equal amounts to 1 ml. lots of freshly prepared 1 to 2% sperm suspensions in sea water. The resulting 2 ml. lots of treated suspension were then fixed by adding to them 3 drops of full strength neutral formalin. A small amount of each such suspension was placed on the formvar film of an electron microscopic grid; excess cells and the sea water were removed by washing with distilled water, and the preparations dried.

Counts of spermatozoa with reacted acrosomes and the total numbers of spermatozoa were made by direct observation. Since the rules governing selection of the fields for such counts were sometimes difficult to obey strictly under the given conditions, a rather large number of counts was made of each sample.

RESULTS

Three series of fixations were made, two of *Pseudocentrotus* and one of *Hemicentrotus* spermatozoa. The first *Pseudocentrotus* experiment was performed early in the breeding season (Nov. 10); the jelly solution, prepared without acidification, gave an agglutination titer of 1,024. This was used in ten-fold dilutions (1/1–1/1,000) for treating freshly shed spermatozoa from three males, and the treated suspensions were fixed with formalin as described above. The stock dry sperm was stored at 10° C. immediately after collection, and the jelly solution kept in a frozen state.

On the following day, the jelly solution gave the same agglutination titer as before, when checked with sperm suspensions made from freshly shed sperm. When fresh suspensions of the stored dry sperm were tested with the same jelly solution, they were found to have lost none of their capacity for agglutination as the result of aging in the cold for 24 hours. A series of fixations similar to that of the previous day was then made to find out whether the capacity for acrosomal reaction is equally impervious to aging.

The second *Pseudocentrotus* experiment was performed 40 days later (Dec. 20), at the height of the breeding season. The jelly solution, made by acid-extraction, had an agglutination titer of 2,048. This was used full-strength and in a series of dilutions; other conditions were the same as those of the earlier experiment.

Fixations of *Hemicentrotus* gametes were made on March 8, near the end of the breeding season, under the same set of conditions as those of the second *Pseudocentrotus* experiment.

The percentages of reacted acrosomes counted with the electron microscope, usually at a magnification of 2,500×, are given in Table 1, and plotted against jelly concentration in Figure 1. COLLIER's (1959) values for *Strongylocentrotus purpuratus* have been added to this figure, with the assumption that his fertilizin titer of 64 corresponds approximately to ours of 256[2].

Curves B (*Pseudocentrotus*) and C (*Hemicentrotus*) are alike in indicating a sharp increase in the number of reacting acrosomes with slight increase in the concentration of jelly substance. A possi-

[2] This much reduction in the value of the endpoint can readily occur if diagnosis of clumping is made with the naked eye.

Table 1. Percentages of reacted acrosomes in fresh and aged sea urchin spermatozoa following treatment with graded concentrations of dissolved jelly substance (fertilizin)

Concentration of egg-water determined by serially diluting stock solution: "1 unit" = smallest amount of jelly substance which will induce sperm agglutination

Units of egg-water	*Pseudocentrotus* Fixed early in season				*Pseudocentrotus* Fixed in mid-season				*Hemicentrotus* Fixed late in season			
	A Fresh dry sperm		A′ Aged dry sperm		B Fresh dry sperm		B′ Aged dry sperm		C Fresh dry sperm		C′ Aged dry sperm	
	Number sperm counted	Percent acrosomes reacted	Number sperm counted	Percent acrosomes reacted	Number sperm counted	Percent acrosomes reacted	Number sperm counted	Percent acrosomes reacted	Number sperm counted	Percent acrosomes reacted	Number sperm counted	Percent acrosomes reacted
0	365	1.6	740	0.7	918	16.2	492	1.8	250	5.2	250	6.0
1	553	4.1	395	5.3	238	27.7	323	3.4	250	16.4	250	6.0
2					321	34.2	331	4.5	250	22.4	250	5.6
4					211	41.2	269	4.4	250	21.6	250	6.8
10	419	4.3	595	6.7								
20					501	57.8	296	8.1	250	26.8	250	6.4
100	351	18.8	628	8.2								
200					582	60.3	343	6.1	250	33.6	250	18.4
1,000	335	23.6	581	22.3								
2,000					651	56.8	313	7.9	250	30.0	250	27.6

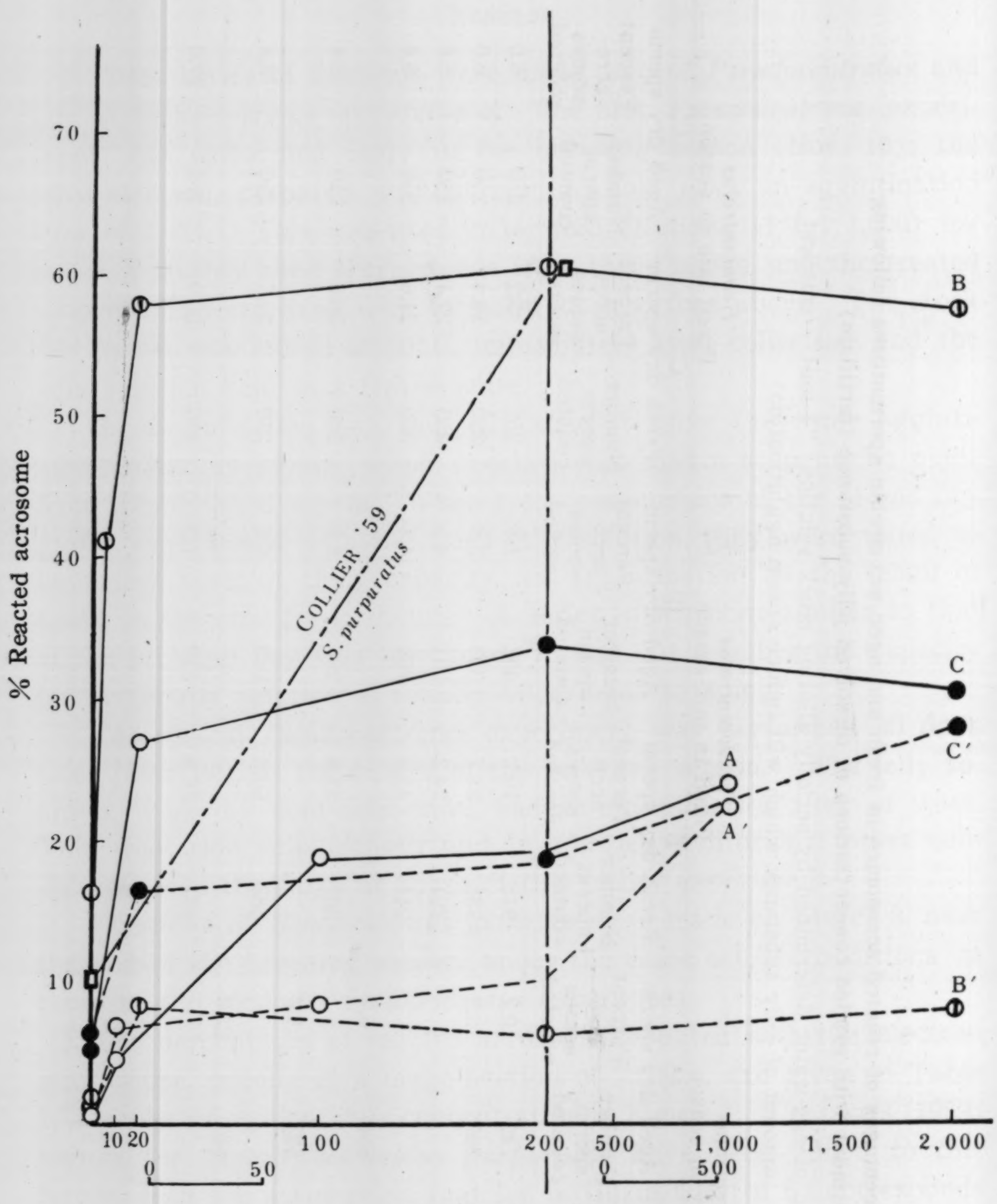

Fig. 1. Reaction of fresh and aged sea urchin spermatozoa to dissolved jelly substance.[1)]

A — *Pseudocentrotus* — early in season
A' — 〃 aged 24 hrs.
B — 〃 — height of season
B' — 〃 aged 24 hrs.
C — *Hemicentrotus*
C' — 〃 aged 24 hrs.

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1) Since the starting points of the curves overlap each other, some of these have been omitted from the figure in the interest of clarity. See Table 1 for data.

ble explanation of the more gradual rise shown by Curve A will be discussed below. In Experiments B and C, as also in the similar set of data for *S. purpuratus* presented in COLLIER's Table 2, a slight falling off of the percentage of reacted acrosomes appears in lots of spermatozoa treated with the most concentrated jelly solution. This effect is not seen in Curve A, or in any of the sets of data obtained with aged spermatozoa (Curves A′, B′, C′). These three sets of data obtained with aged sperm also resemble each other and those of Experiment A in failing to respond to traces of jelly substance in solution.

DISCUSSION

These results bring to light one fact of considerable significance for the main purpose of this study: that sea urchin spermatozoa in "good" condition respond in something like an all-or-none manner to trace amounts of jelly substance in sea water solution, by a reaction of the acrosome (Experiments B and C). This is important because it seems obvious that the amount of such dissolved jelly must be very small in the vicinity of freshly spawned eggs when fertilization occurs under natural conditions (see also TYLER, 1948). That, however, this small amount is highly effective in evoking the acrosome reaction has been shown by the thin-section studies of AFZELIUS (1956) and AFZELIUS and MURRAY (1957), who found that *all* the spermatozoa "swarming" around eggs fixed 2–4 minutes after insemination had undergone a reaction of the acrosome. From the standpoint of the meaning of the acrosome reaction for fertilization, AFZELIUS's method more closely approaches the natural condition than does one which involves treating the spermatozoa in suspension with artificially dissolved jelly substance, and there is therefore no great logical necessity to account for the appearance in our counts, and the absence from his, of large numbers of spermatozoa which do not respond to the presence of egg jelly in solution. It may, however, be suggested that this discrepancy between AFZELIUS's apparent 100% reactivity and the maximum of about 60% found by COLLIER and ourselves points to the presence, in any suspension, of spermatozoa which are physiologically substandard to such an extent that they do not even approach the egg surface. There is also a possibility in AFZELIUS's experiment that spermatozoa with reacted acrosomes remained at-

tached to the egg surface while unreacted spermatozoa were removed during the fixing and dehydrating procedures.

The often-invoked idea that there are differences in physiological condition and reactivity among the sperm cells in any spawning rests on a rather vague basis of common sense probability, the absence of contrary evidence, and the necessity to account in some way for the considerable fluctuations commonly found in tests of fertilizability. In the present study, also, the marked contrast between the results of the two *Pseudocentrotus* experiments suggests that some, as yet not clearly definable, lack of "physiological maturity" characterizes the spermatozoa obtained early in the breeding season (Experiment A), to the extent that the percentage of reacted acrosomes found in the control is only one-tenth that of the figure obtained at the height of the season (Experiment B), and the maximum amount of reaction induced in the "immature" suspension by the most concentrated jelly solution (23.6%) is less than that occurring at a 1/1,000 dilution in the "mature" spermatozoa (27.7%).

The effect of aging would also be expected to make its first appearance as a loss of reactivity, especially to a minimal stimulus. This expectation is supported by the data arranged in Curves A′, B′ and C′. The percentages of reacted acrosomes in these suspensions of aged spermatozoa fail to show any significant increase in the low-concentration range of the jelly solutions, although in Experiments A and C the proportion of reacted acrosomes is nearly the same in both fresh and aged suspensions treated with the most concentrated solutions.

So far as these data go, they show positive correlations between acrosome reactivity and optimum part of the breeding season (Curve B *vs.* Curve A), as well as "freshness" of the sperm cells (Curves A, B and C *vs.* A′, B′ and C′). The results of the experiment with *Hemicentrotus*, however, are equivocal and inadequate, although not entirely without interest, since they reflect the tendencies of the *Pseudocentrotus* data.

As reported above, aging of the spermatozoa for 24 hours was found to have no effect on their capacity for agglutination. In other words, they formed clumps even in the highly diluted jelly solutions exactly as they had immediately after being shed. Their failure, then, to undergo acrosome reaction to a corresponding extent provides

further evidence (*v.* DAN, 1954) for considering the phenomena of agglutination and acrosome reaction as two separate responses of sperm cells to the presence of egg jelly substance.

SUMMARY

Spermatozoa of the sea urchins *Pseudocentrotus depressus* and *Hemicentrotus pulcherrimus* were exposed to graded concentrations of dissolved egg jelly substance, and samples examined under an electron microscope for occurrence of the acrosome reaction. Suspensions were made from dry sperm both freshly shed and after aging at 10° for 24 hours.

The freshly shed spermatozoa showed a tendency to respond, if at all, to very small amounts of dissolved jelly substance within the range of 10–20 times the minimum concentration effective in causing agglutination. Increasing the jelly concentration further than this did not induce correspondingly more spermatozoa to react. Aged spermatozoa conspicuously failed to respond to minimum amounts of jelly substance, although in some cases the percentages of acrosomes reacting to high concentrations were similar to those of freshly suspended cells. In contrast to this difference in the pattern of the acrosome reaction, aging for 24 hours caused no detectable change in the agglutinating capacity of the spermatozoa.

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VEGETALIZATION OF THE PRESUMPTIVE ECTODERM OF THE *TRITURUS*-GASTRULA BY EXPOSURE TO LITHIUM CHLORIDE SOLUTION

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In the sea urchin's egg the treatment with lithium favors the development of the organs normally formed by the vegetal hemisphere of the egg at the expense of the development of animal organs (RUNNSTRÖM, 1928; LINDAHL, 1936; etc.). On the other hand, in the amphibian embryo lithium was found to suppress the notochord formation, or to reduce the neural structures including optic rudiments (LEHMANN, 1937, 1938; TÖNDURY, 1938; ÔGI, 1954). Thus the lithium effect on the sea urchin's embryo, which is commonly referred to as vegetalization, is not easily brought into unity with that on the amphibian embryo, for which terms such as mesodermalization (LEHMANN, 1938) or ventralization (BÄCKSTRÖM, 1954) have been proposed. In order to analyse the apparently complex effect of lithium on the amphibian embryo, the method of explantation was introduced, leading to some significant results (HALL, 1942; LOMBARD, 1952; MASUI, 1956, 1959, 1960 a, b, c). Special attention should be given to the recent experiments of MASUI who combined the isolated organizer and the isolated presumptive ectoderm, both of which were either treated or not treated beforehand with lithium solution, and arrived at the following conclusions: (1) The effect of lithium on the organizer consists chiefly in a suppression of the differentiation of the notochord. (2) The reaction pattern of the presumptive ectoderm toward a stimulus which ordinarily is the archencephalic differentiation is modified by lithium to the deuterencephalic and spino-caudal one. This effect of lithium found by MASUI may be

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interpreted as a caudal shift in the reaction pattern of the ectoderm.

In the present experiments the isolated presumptive ectoderm was first treated with lithium chloride solution and then exposed to a non-living inductor which is known to cause archencephalic structures in the untreated ectoderm (Series I-B). As such an inductor the heat-treated guinea pig liver was used. A comparison of the results of this series with those of the control series, in which untreated ectoderm was exposed to the same inductor (Series I-A), was made in order to check the possible shift in the regional inductive effect. In an other series of experiments differentiation of the isolated presumptive ectoderm treated with lithium chloride but not exposed to the inductor was studied (Series II-A～D).

Material and Methods

A piece of the presumptive ectoderm, measuring *ca.* 1 mm. in diameter, was isolated from the earliest gastrula of *Triturus pyrrhogaster*, and was treated for 4 hours in lithium chloride solution prepared as described below. In Series I, after thorough washing for about one hour in Holtfreter's solution, the treated isolates were exposed to a piece of liver tissue, *ca.* 0.4 mm. in diameter, by the sandwich technique, and were cultured in Holtfreter's solution for 11–14 days. The liver tissue was removed from starved guinea pig and kept in 70% alcohol and then was boiled for about 5 minutes at *ca.* 70° C. immediately before use. In the control series, the untreated ectoderm was cultured with a piece of the liver tissue in the same way.

In Series II, the lithium-treated ectoderm was cultured alone in Holtfreter's solution adjusted to pH 7.2-7.4 with sodium bicarbonate or Tris.

The lithium solution used was prepared as follows: 1 M aqueous stock solution was diluted with Holtfreter's solution buffered with sodium bicarbonate or water to the desired concentration. The operation was done in the sterile condition. The explants thus obtained were cultured for 11 to 14 days at 19° C. in sterile Holtfreter's solution. The sections were stained with Mayer's hemalum and picroblauschwarz for histological observation.

Results

Series I. Differentiation of the presumptive ectoderm treated or not treated with lithium and cultured with the liver tissue.

The isolates were treated for 4 hours with M/16 or M/50 lithium chloride diluted with HOLTFRETER's solution. During the treatment the isolates tend to curl inside out. After transfer to HOLTFRETER's solution they recovered the original shape. They were cultured

Table 1. Differentiation of the ectoderm treated with lithium and then cultured with the liver tissue (Series I).

No. of Series	I-A	I-B	I-C
Reacting system	Untreated ectoderm	Treated ectoderm	
		M/16 LiCl (pH 7.2)	M/50 LiCl (pH 7.2)
No. of valid explants	82	40	36
No. of explants with any induction	76(93%)	38(95.0%)	32(88.9%)
Eye (pigment vesicle)	32(39.0%)	1(2.5%)	2(5.6%)
Fore-brain	35(42.3%)	1(2.5%)	2(5.6%)
Nose	17(20.7%)	1(2.5%)	1(2.8%)
Lentoid	1(1.2%)	0	0
Non-specifiable brain	6(7.3%)	0	2(5.6%)
Non-specifiable neural tissue	14(17.1%)	1(2.5%)	4(11.1%)
Hind-brain	0	2(5.0%)	2(5.6%)
Ear vesicle	0	2(5.0%)	0
Spinal cord	0	5(12.5%)	6(16.7%)
Melanophores	28(34.1%)	13(32.5%)	12(33.3%)
Notochord	0	4(10.0%)	0
Somites	0	13(32.5%)	4(11.1%)
Nephric tubules	0	7(17.5%)	1(2.8%)
Mesothelium	0	5(12.5%)	4(11.1%)
Blood island	0	6(15.0%)	3(8.3%)
Mesenchyme	33(40.2%)	33(82.5%)	27(75.0%)
Endoderm-like mass	0	4(10.0%)	1(2.8%)
No. of neural induction	58(77.4%)	7(17.5%)	14(38.9%)
No. of archencephalic induction	38(46.2%)	1(2.5%)	3(8.3%)
No. of deuterencephalic induction	0	2(5.0%)	3(8.3%)
No. of spino-caudal induction	0	5(12.5%)	7(19.4%)
No. of trunk-mesodermal induction	0	22(55.0%)	10(27.8%)

together with the liver tissue in a sandwich. In the control series, the sandwich was prepared with the untreated ectoderm and liver tissue, and cultured under the same conditions.

As indicated in Table 1, the untreated ectoderm (Series I-A)

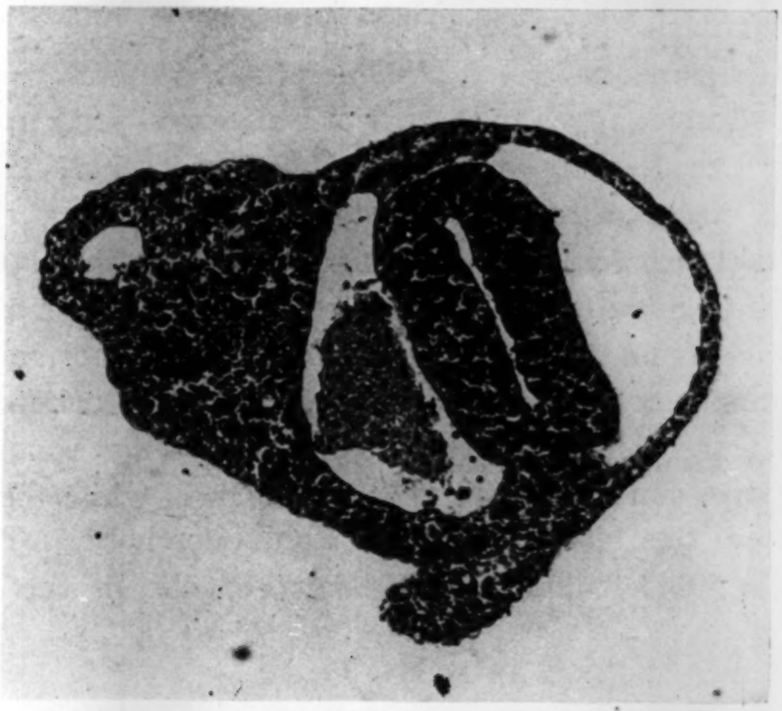

Fig. 1

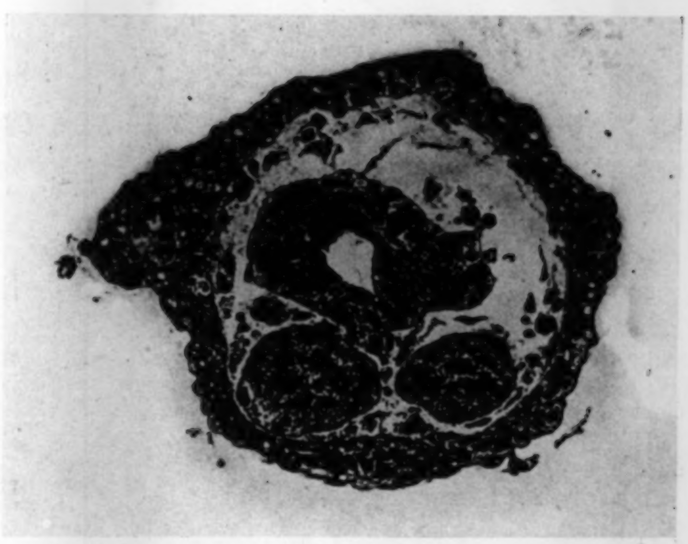

Fig. 2

Fig. 1 and 2. Archencephalic induction in the untreated ectoderm exposed to the inductive influence of liver tissue. Fig. 1: Fore-brain type, Fig. 2: eye-type. Liver tissue in the middle. ×100.

was induced by the liver tissue to form the archencephalic structures such as eye, fore-brain, and nose. Melanophores, mesenchyme were also noted (Figs. 1 and 2). On the contrary, in the Series I-B, C with treated ectoderm, a tendency was observed to differentiate the more posterior neural structures, such as hind-brain and spinal cord (Fig. 3), together with ear-vesicles. The tendency was accompanied by significant suppression of archencephalic structures. Simultaneously with those changes in the pattern of neural structures, the appearance of mesodermal structures, including notochord, somite, nephric tubules, mesothelium and blood island, was noted (Figs. 4, 5). So far as microscopic study of sections indicates these mesodermal tissues were in a well advanced stage of differentiation and were not distinguishable from those produced by the isolated normal mesoderm under a similar culture condition. Out of 4 notochords listed in Table 1, 3 were slender and composed of weakly vacuolated cells suggesting the condition of the tail-notochord, while the remaining one indicated the features of the trunk-notochord. The somites shown in the table included spindle-formed cells of the early myo-

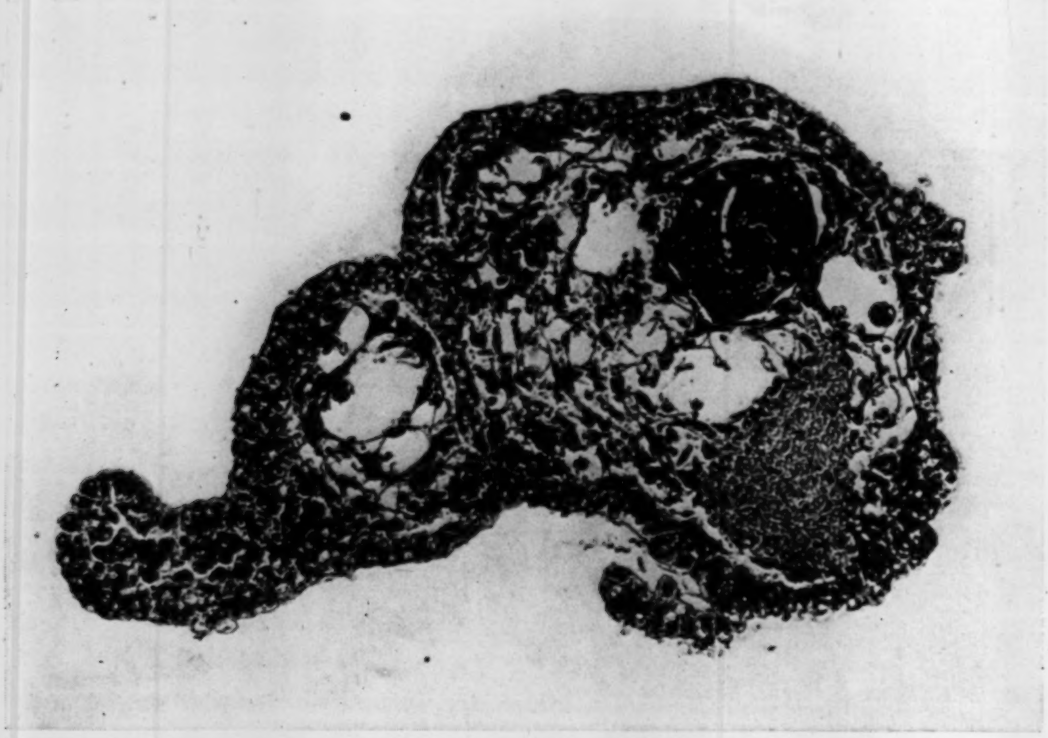

Fig. 3. Spinal cord-type induction accompanied by mesenchyme and melanophores in the treated ectoderm (M/50, LiCl) under the influence of liver tissue. Right below, liver tissue. Right above, spinal cord. ×100.

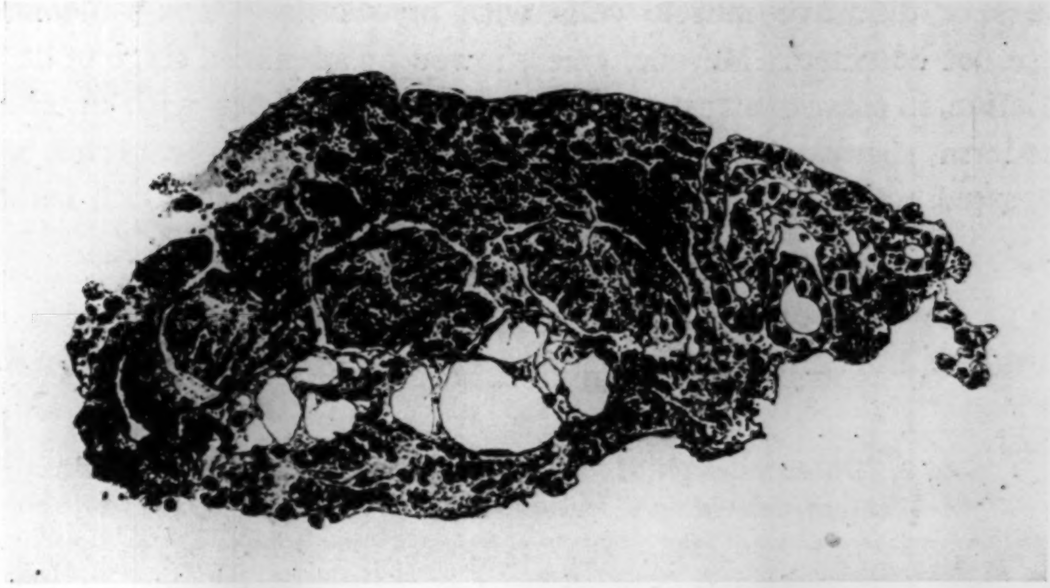

Fig. 4. Trunk-mesodermal structures obtained in the treated ectoderm (M/16, LiCl) exposed to liver tissue. Notochord (below), somites (middle), nephric tubule (right) are visible. Spinal cord is also found in the left side. ×100.

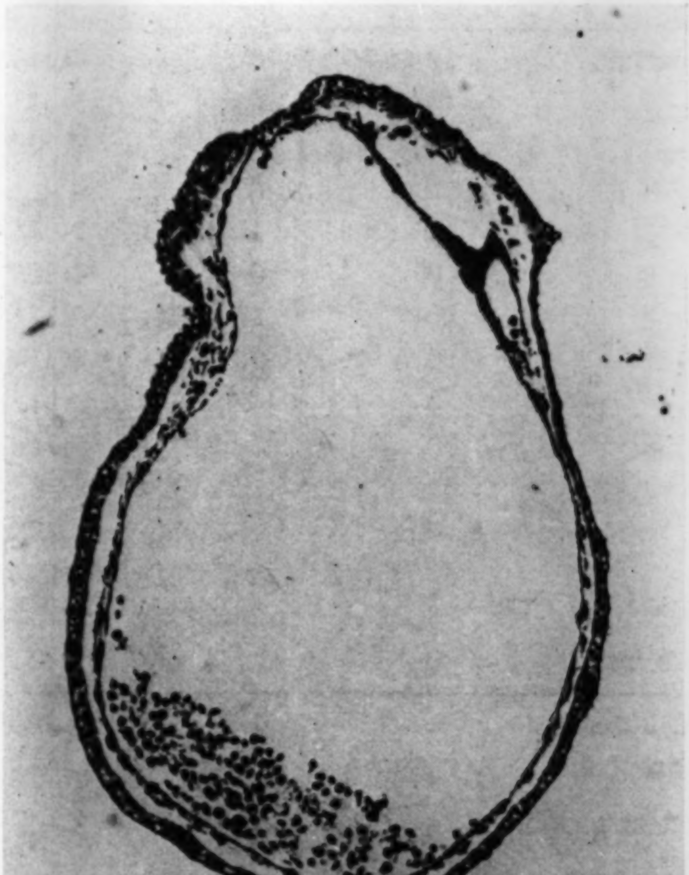

Fig. 5. Mesothelium, blood island and mesenchyme differentiated in an explant of the same series as illustrated by Fig. 4. Liver tissue is not visible. ×70.

tomes or difinitive muscle cells with myofibrils. The tail-somites were not detected. Mesenchyme showed an advanced stage of differentiation, in clear contrast to that found in the series with untreated ectoderm (Series I-A). Another distinction from the series with untreated ectoderm was the presence of a mass of yolk-rich cells.

Series II. Differentiation of the lithium-treated ectoderm cultured without the liver tissue.

The concentrations of lithium chloride solution used in this series

Table 2. Differentiation of the presumptive ectoderm treated with lithium chloride and cultured in isolation (Series II).

No. of Series	II-A	II-B	II-C	II-D
Concentration of LiCl	M/16 (pH 7)	M/16 (pH 7.2)	M/16 (pH 8.6)	M/50 (pH 8.6)
Diluted with	Water	HOLTFRETER's solution		
Valid explants	15	35	34	80
Atypical epidermis	10(66.7%)	10(28.5%)	4(11.7%)	55(68.9%)
Eye	0	0	1(2.9%)	0
Fore-brain	0	0	2(5.9%)	2(2.5%)
Nose	0	0	0	1(1.3%)
Neural tube	0	1(2.9%)	1(2.9%)	0
Neural tissue	0	0	4(11.7%)	1(1.3%)
Melanophores	0	1(2.9%)	6(17.6%)	0
Notochord	0	1(2.9%)	5(14.4%)	0
Somites	0	5+2?(20.0%)	8(23.5%)	0
Nephric tubules	0	1(2.9%)	5(14.4%)	0
Mesothelium	0	1(2.9%)	3(8.8%)	1(1.3%)
Blood island	0	4(11.4%)	1(2.9%)	2(2.5%)
Mesenchyme	1(6.7%)	20(57.2%)	20(58.8%)	20(25.0%)
Indication of mesenchyme formation	4(26.7%)	2(5.7%)	3(8.8%)	4(5.0%)
Endoderm-like mass	0	0	1(2 9%)	0
No. of neural induction	0	1(2.9%)	6(17.6%)	3(3.8%)
No. of archencephalic induction	0	0	2(5.9%)	2(2.5%)
No. of deuterencephalic induction	0	0	0	0
No. of spino-caudal induction	0	1(2.9%)	1(2.9%)	0
No. of trunk-mesodermal induction	0	11(31.4%)	12(35.3%)	2(2.5%)

were M/16 and M/50. Its pH was adjusted to 7.2 or 8.6. In two or three hours after the transfer into the culture medium, explants assumed a spherical shape and were easily cultured. The results of histological observation differed according to whether pure water or HOLTFRETER's solution was used to make up the lithium chloride solution (Table 2). The differentiation of the ectoderm treated with the lithium chloride-HOLTFRETER's solution was characterized by the occurrence of mesodermal structures (Fig. 6, 7) as well as mesenchyme (Series II-B, C, D). In thses cases, at the higher concentration of lithium chloride or at the higher pH value the trunk-mesodermal structures occured at higher percentages. Only in Series II-C, one undifferentiated endoderm-like mass was encountered. It should also be notice that archencephalic neural structures were encountered in a few cases in the series of high pH value (Series II-C, D). Furthermore, in all 4 groups of this series, within the epidermal cells a peculiar condensation of cells with conspicuous stainability was observed. These cases seem to show an intermadiate condition between epidermal and mesenchymal differentiation, and they are summarized in Table 2 as "indication of mesenchyme formation". This condition was also observed in the presumptive ectoderm treated with aqueous lithium solution.

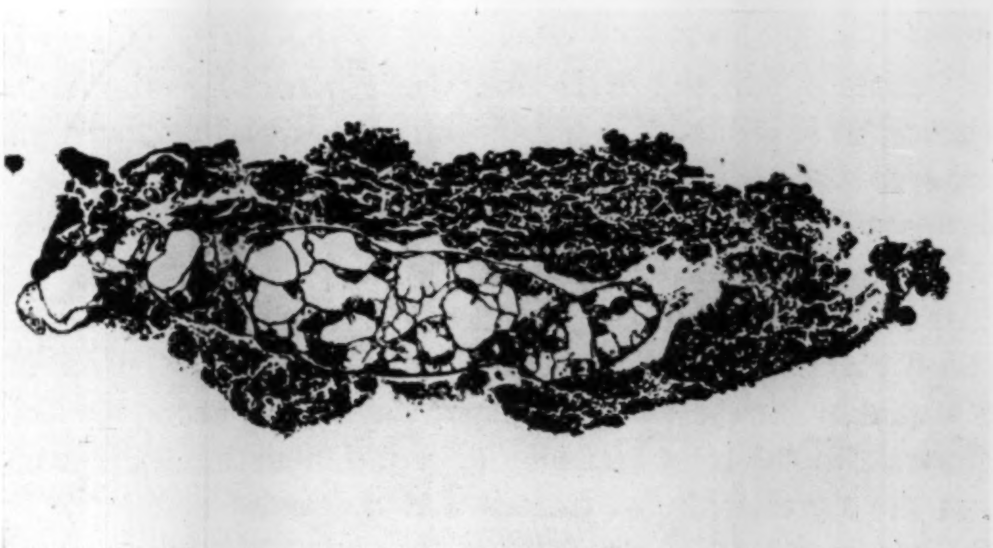

Fig. 6. Notochord-type structure differentiated in the ectoderm treated with M/16 Li-HOLTFRETER's solution at pH 8.6, without being exposed to liver tissue. ×100.

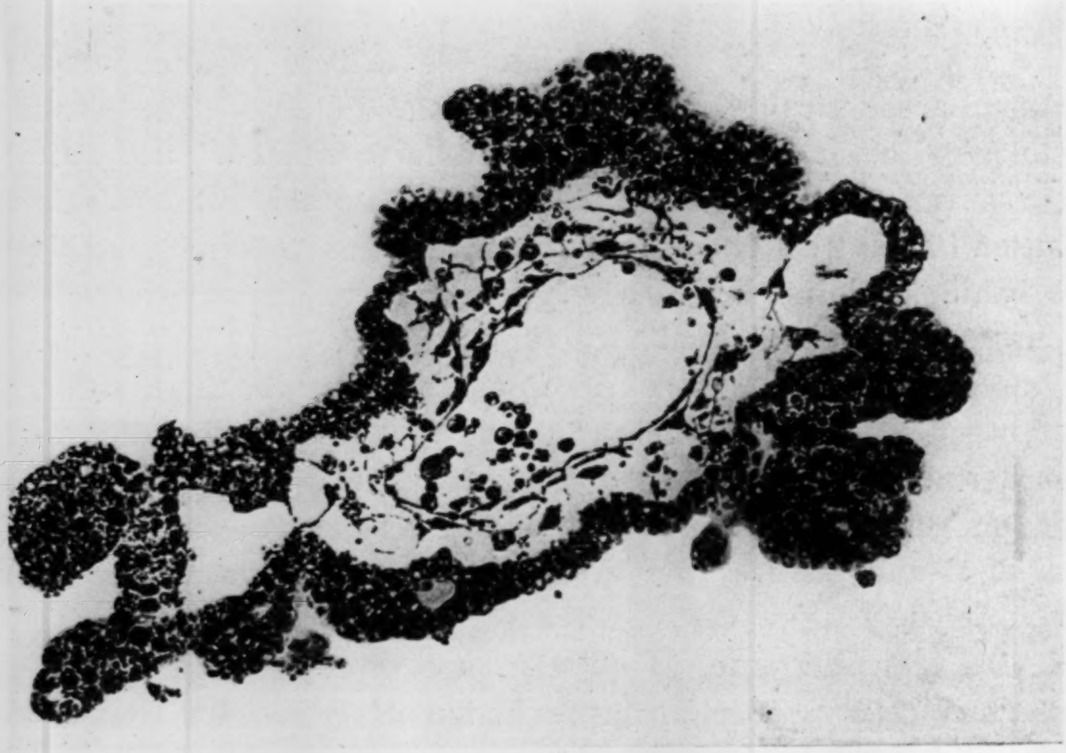

Fig. 7. Mesothelium, blood island and mesenchyme formed in an explant of the same series as illustrated by Fig. 6. ×100.

Discussion

That the untreated ectoderm gives rise to archencephalic structures under the influence of liver tissue is consistent with the earlier reports of Toivonen (1940). However, in the series with lithium-treated ectoderm exposed to the liver tissue (Series I-B) the frequency of archencephalic structure is significantly lower, and deuterencephalic and spino-caudal structures are observed contrary to the cases with the untreated ectoderm (Series I-A). The result confirms the suggestion made in the introduction that lithium-treatment may lead to a caudal shift of the reaction pattern of the ectoderm. Similar results have been obtained by Masui (1959) who studied the effect of the living organizer on the lithium-treated ectoderm. From the fact that no difference in the total fequency of neural induction was recognized between the series with the treated and untreated ectoderm, Masui (1960 b) has been lead to the opinion that the lithium-treatment does not suppress the neuralization of the ectoderm. In the present case, however, the reduction of the neural differentiation by the lithium-treatment of the reacting ectoderm is clearly evidenced. The dis-

crepancy will be discussed in a later section.

What should be emphasized in the present results is the occurrence of mesodermal differentiation in the treated ectoderm cultured either with or without the inductor. In the treated ectoderm without the inductor (Series II), the highest frequency of mesodermal structures was observed at the higher pH value, and at the higher concentration of lithium chloride used. The fact that the treatment with aqueous lithium solution caused only very slight effect could be interpreted by assuming a supplementary role of other ions contained in HOLTFRETER's solution in the morphogenetic effect of lithium ion (cf. ELBERS, 1952; RAVEN, 1956). For understanding the physicochemical mechanism of the lithium effect further study of the phenomenon appears to be needed.

Further suggestive is the fact that groups of yolk-rich cells are found among mesodermal tissues of the explant in the Series I and II. The condition is similar to that observed in the sandwich explant in which the untreated ectoderm was combined with guinea pig bone marrow which is noted for the mesoderm-inducing ability (TAKATA, C. and YAMADA, 1960). In this case yolk-rich cells were found among induced mesodermal structures which, upon a long culture, differentiated into definitive endodermal tissues. Appearance of yolk-rich cells and mesodermal structures out of the lithium-treated ectoderm in the present experiments may be interpreted as vegetalization of the developmental pathway of the ectoderm, since on the surface of the blastula the presumptive mesoderm and presumptive endoderm lie more vegetally than the presumptive ectoderm. Thus we may have here a vegetalization of the amphibian germ layer comparable to the vegetalization of the animal hemisphere of the sea urchin's embryo.

A mesodermal differentiation of the isolated ectoderm of *Triturus pyrrhogaster* treated with lithium chloride in a slightly alkaline medium (pH 8.2) has been evidenced by MASUI (1960 b). Further, BARTH and BARTH (1959) reported that the cells of the presumptive epidermis of *Rana pipiens* gastrulae treated with lithium differentiated mesenchyme and occasionally notochord-like cells.

Comparing Series I and II, it can be recognized that the presence of inductor increases the frequency of mesodermal structures, especially the frequecy of the dorsal mesodermal structures. This is most

clearly demonstrated by comparing Series I-B with Series II-B, in which pH value and lithium concentration were comparable. This increase in mesodermal differentiation owing to the presence of inductor is also apparent in the result of MASUI's experiment (MASUI, 1960 b). Further by comparing Series II-C with II-B, a slight enhancement of dorsal mesodermal structures appears to be caused by a higher pH value used in the lithium-treatment of the ectoderm. These facts are compatible with the idea that higher pH value and liver tissue have a dorsalizing effect (YAMADA, 1950; KARASAKI, 1957): The differentiation pattern of the ectoderm is switched from epidermal to neural, and that of mesoderm from ventral to dorsal.

It should be further pointed out that the vegetalization of the ectoderm may itself be the cause of the caudal shift of neural pattern observed in the treated ectoderm exposed to the archencephalic inductor, since the cranial end of the neural system is derived from the animal pole region, and the caudal end of the neural system is derived from the more vegetal region of the ectoderm. From the decrease in neural frequency by the lithium-treatment (compare Series I-A with I-B, C), if the ectoderm is too much vegetalized, it may be expected that the ectoderm differentiates into more mesodermal or endodermal tissues. However, in weak vegetalization, regionality will be shifted within neural tissue from archencephalic to deuterencephalic and spino-caudal. Such may also be the case in MASUI's experiments (1960 b). In this sense lithium acts like the cephalo-caudal mediator proposed by YAMADA (1950, 1955) in his theory of the embryonic organization.

At the present stage of investigation although one should not claim that all morphogenetic effects of lithium on the amphibian embryo can be interpreted as vegetalization, the general trend of the results presented here implies that there is a common principle operative in the primary embryonic organization between amphibians and echinoderms.

SUMMARY

1. The differentiation of the isolated presumptive ectoderm of the *Triturus gastrula* under the influence of an archencephalic inductor (liver tissue of the guinea pig) was compared between presumptive ectoderm with and without pre-treatment with lithium solution. In

the latter series the archencephalic type of differentiation was dominating, in the former series the trunk-mesodermal type appeared together with spino-caudal and deuterencephalic types.

2. The trunk-mesodermal structures were also formed from the ectoderm treated with lithium-HOLTFRETER's solution alone not exposed to the liver tissue.

3. Mesodermal tendency precieved in the ectoderm and caudal shift in the neuralization pattern of the ectoderm induced by the lithium-treatment were interpreted as vegetalization of the ectoderm.

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EMBRYOLOGIA, Vol. 5, No. 4, pp. 397-405 (March, 1961)

STUDIES ON THE PRODUCTION OF THE FERTILIZATION ACID IN SEA URCHIN EGGS

I. ACID PRODUCTION AT FERTILIZATION AND ACTIVATION, AND THE EFFECT OF SOME METABOLIC INHIBITORS

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INTRODUCTION

In recent years, considerable progress has been achieved in the field of fertilization physiology in Metazoa. Nevertheless, there remain many problems unsolved, one of which is the production of the fertilization acid in sea urchin eggs. In 1929 ASHBEL discovered, using manometric technique, that the eggs of *Arbacia lixula* evolved carbon dioxide suddenly after fertilization. RUNNSTRÖM (1930, 1933) also observed the same phenomenon with *Paracentrotus lividus* and examined it in detail. The carbon dioxide is derived from the bicarbonate present in the surrounding sea water, following the diffusion of an acid out of the egg. Such a phenomenon has also been reported by several other workers (BOREI, 1933; ÖRSTRÖM, 1935; LASER and ROTHSCHILD, 1939 and ALLEN, MARKMAN and ROWE, 1958).

The present author (1957) observed a transient accumulation of lactic acid inside eggs following fertilization in the sea urchins, *Hemicentrotus pulcherrimus*, *Anthocidaris crassispina* and *Pseudocentrotus depressus*, and suggested a possibility that this accumulation might be related, either directly or indirectly, to the acid production. ROTHSCHILD (1958) re-examined this possibility with eggs of *Echinus esculentus*, but could not observe the production of lactic acid. Thus, because of the failure in generalizing this phenomenon, an attempt as to correlate the production of the fertilization acid with that of the lactic acid was obliged to be abandoned. Moreover, it has not yet been settled whether the acid production is related to some metabolic change in respiration or glycolysis at fertilization. Some

other possibilities were presented (ROTHSCHILD, 1956; RUNNSTRÖM, 1959).

The chemical nature of the acid is also unknown. It is neither lactic, pyruvic, nor malic acid and, according to YČAS (1950), it is unlikely to be any of the KREBS cycle acids.

For the purpose of solving this long-standing problem, a series of experimental works has been undertaken. The present paper will give some data on the acid production of fertilized and activated sea urchin eggs, together with those on the effect of some metabolic inhibitors on the acid production.

The author wishes to thank Prof. T. YAMAMOTO and Dr. E. NAKANO for their helpful advice and encouragement. Thanks are also due to the Director of the Sugashima Marine Biological Station, Prof. M. SUGIYAMA and to his staff for making facility for carrying out the work and for the valuable criticism.

MATERIAL AND METHOD

Materials used were regular sea urchins, *Hemicentrotus pulcherrimus, Pseudocentrotus depressus, Temnopleurus toreumaticus* and *Anthocidaris crassispina*, collected at the Sugashima Marine Biological Station. Shedding of eggs was made by pouring isotonic KCl solution into the body cavity. The shed eggs were repeatedly washed with filtered sea water. If necessary, eggs were rendered jelly-free by the treatment with the isotonic NaCl solution containing 10^{-4} M EDTA (ethylene diamine tetra-acetate).

The production of the fertilization acid was observed by measuring the fall in pH of the surrounding sea water with the electric pH-meter of Toa Dempa Co. Ltd. This method was first employed by ALLEN *et al.* (1958) in eggs of *Arbacia punctulata* and *Psammechinus miliaris.*

The procedure of the artificial activation will be described in the following section.

RESULTS

Acid production at fertilization

15 ml. of dense suspension of eggs was mixed with 5 ml. of sperm suspension (10^{-4}). Mixing was performed as quickly as possible

allowing nearly synchronous insemination, and changes in pH of the suspension were periodically followed. Throughout the measurement the suspension was continuously stirred. A typical result with each species is presented in Fig. 1. It can be seen in the figures that the acid production starts shortly after insemination and continues for a while, about 2 to 3 minutes according to the materials used. The dissolution of the jelly coat, which occurs at fertilization in the present materials, did not affect the fall in pH of the sea water. Control suspensions of unfertilized eggs or sperm alone affected the pH of the sea water only slightly over an extended period.

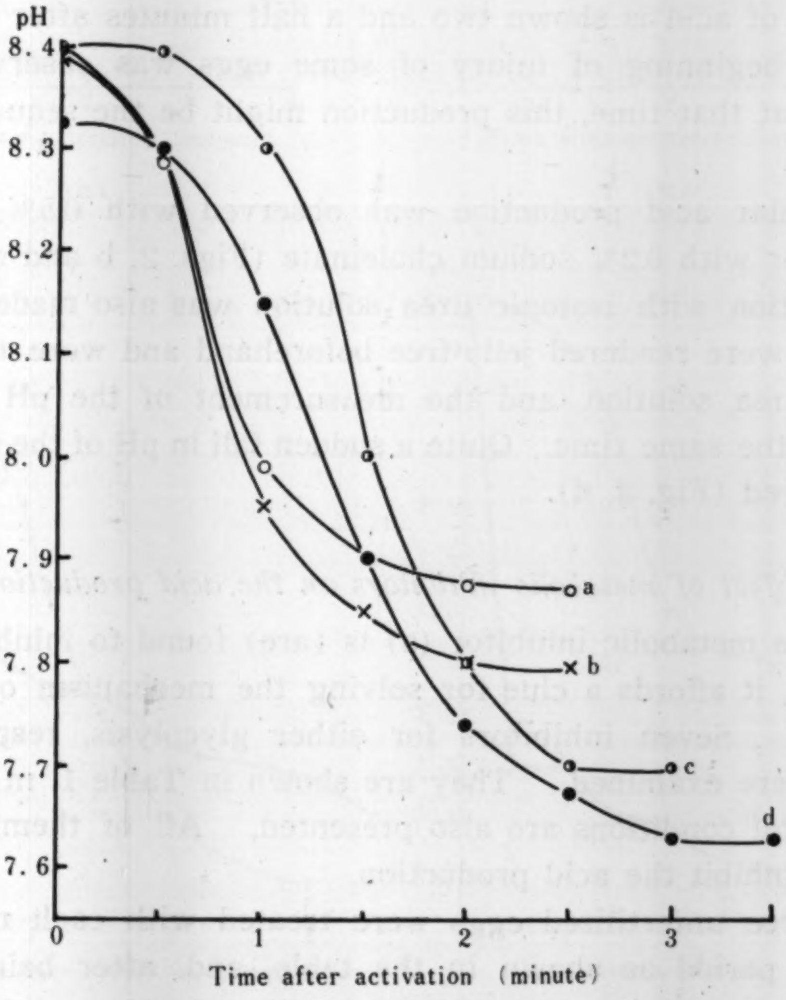

Fig. 1. Acid production following fertilization.

(a) *Anthocidaris crassispina.*
(b) *Temnopleurus toreumaticus.*
(c) *Pseudocentrotus depressus.*
(d) *Hemicentrotus pulcherrimus.*

Acid production at activation

When the sea urchin eggs are treated with sea water containing suitable concentration of sodium choleinate, (SUGIYAMA, 1953 a) or the detergent, Monogen or Lipon (SUGIYAMA, 1953 b) or thymol (ISHIKAWA, 1954), breakdown of the cortical granules and elevation of the fertilization membrane take place.

Into 14 ml. of egg suspension 6 ml. of thymol-sea water (thymol-saturated sea water, 3 parts; sea water, 7 parts) was added, followed by rapid stirring of the suspension. Rapid and full elevation of the membrane took place, accompanied with the rapid fall in pH of the medium. One example is shown in Fig. 2, a. In the figure, secondary production of acid is shown two and a half minutes after activation. Since the beginning of injury of some eggs was observed microscopically at that time, this production might be the sequence of the egg injury.

A similar acid production was observed with 0.5% Lipon or Monogen or with 0.2% sodium choleinate (Figs. 2, b and c).

Activation with isotonic urea solution was also made. In this case, eggs were rendered jelly-free beforehand and were transferred into the urea solution, and the measurement of the pH shift was started at the same time. Quite a sudden fall in pH of the suspension was observed (Fig. 2, d).

Effect of metabolic inhibitors on the acid production

If some metabolic inhibitor (s) is (are) found to inhibit the acid production, it affords a clue for solving the mechanism of the acid production. Seven inhibitors for either glycolysis, respiration or esterase were examined. They are shown in Table 1, in which the experimental conditions are also presented. All of them, however, could not inhibit the acid production.

Jelly-free unfertilized eggs were treated with each reagent for respective period as shown in the table, and, after being washed with normal sea water, they were inseminated. Normal fertilization membrane was formed, and normal production of the acid was observed without exception.

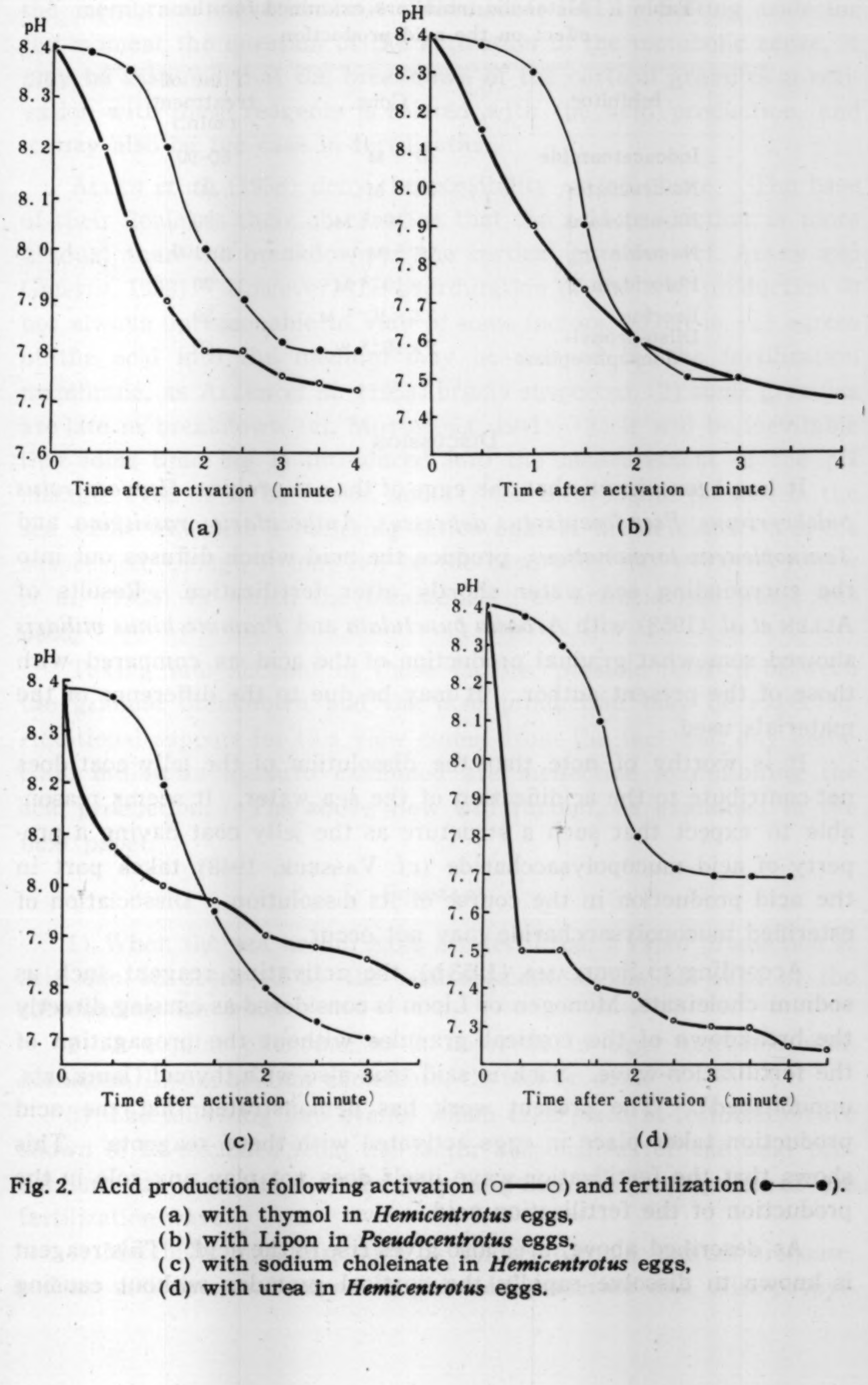

Fig. 2. Acid production following activation (○——○) and fertilization (●——●).
(a) with thymol in *Hemicentrotus* eggs,
(b) with Lipon in *Pseudocentrotus* eggs,
(c) with sodium choleinate in *Hemicentrotus* eggs,
(d) with urea in *Hemicentrotus* eggs.

Table 1. Metabolic inhibitors examined for the effect on the acid production

Inhibitor	Conc.	Time of treatment (min.)
Iodoacetoamide	10^{-3} M	60–90
Na-arsenate	10^{-1} M	90
Na-malonate	2×10^{-2} M	90
Na-azide	10^{-3} M	30–100
Phloridzin	5×10^{-3} M	90
Eserine	2.5×10^{-3} M	30
Diiso-propyl-fluorophosphate	2×10^{-4} %	60

Discussion

It has been shown that the eggs of the sea urchins, *Hemicentrotus pulcherrimus, Pseudocentrotus depressus, Anthocidaris crassispina* and *Temnopleurus toreumaticus*, produce the acid which diffuses out into the surrounding sea water shortly after fertilization. Results of Allen *et al.* (1958) with *Arbacia punctulata* and *Psammechinus miliaris* showed somewhat gradual production of the acid as compared with those of the present author. It may be due to the difference of the materials used.

It is worthy of note that the dissolution of the jelly coat does not contribute to the acidification of the sea water. It seems reasonable to expect that such a structure as the jelly coat having a property of acid mucopolysaccharide (cf. Vasseur, 1948) takes part in the acid production in the course of its dissolution. Dissociation of esterified mucopolysaccharide may not occur.

According to Sugiyama (1953b), the activating reagent such as sodium choleinate, Monogen or Lipon is considered as causing directly the breakdown of the cortical granules without the propagation of the fertilization-wave. Such is said true also with thymol (Ishikawa, unpublished). The present work has demonstrated that the acid production takes place in eggs activated with these reagents. This shows that the fertilization-wave itself does not play any role in the production of the fertilization acid.

As described above, urea also gives rise to the acid. This reagent is known to dissolve rapidly the cortical granules without causing

the membrane elevation in it (MOTOMURA, 1941). Setting aside for the moment the question of the activation in the metabolic sense, it may be assumed that the breakdown of the cortical granules at activation with these reagents is related with the acid production, and it may also be the case in fertilization.

ALLEN *et al.* (1958) deny the possibility stated above. The base of their denial is their observation that the acid production is more gradual than the breakdown of the cortical granules (cf. ALLEN and GRIFFIN, 1958). However, the overduration of the acid production is not always unreasonable in view of some factors. That is, (1) egress of the acid into the medium may be delayed by the fertilization membrane, as ALLEN *et al.* (1958) briefly suspected, (2) some granules are late in breakdown (cf. MOTOMURA, 1941), (3) it will be inevitable that some time lag is introduced into the measurement of the pH change, even if it be minor, and (4) the bicarbonate present in the sea water will have a buffering action against the acidification of the suspension, though it may not be the case with the work of ALLEN *et al.* (1958) in which the bicarbonate-free artificial sea water was used.

Taking into account of these factors, possible relation between the granule breakdown and the acid production may be expected. Additional support for this view comes from the fact that any metabolic inhibitors hitherto examined are ineffective in inhibiting the acid production. The above view will further be examined in the next paper.

SUMMARY

1) When the sea urchin eggs are fertilized, a rapid production of acid can be observed by the measurement of the pH shift of the surrounding sea water.

2) A similar phenomenon is observed in eggs activated with Monogen, Lipon, sodium choleinate, thymol or urea.

3) The following two events which take place at fertilization are shown to be excluded from the factor responsible for the acid production; (a) dissolution of the jelly coat and (b) propagation of the fertilization-wave.

4) Metabolic inhibitors such as iodoacetoamide, sodium arsenate, sodium malonate, sodium azide, phloridzin, eserine and diiso-propyl-

fluorophosphate are all proved to be ineffective in inhibiting the acid production.

5) A possibility is suggested that the breakdown of the cortical granules following fertilization may be related with the acid production.

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EMBRYOLOGIA, Vol. 5, No. 4, pp. 408–414 (March, 1961)

STUDIES ON THE PRODUCTION OF THE FERTILIZATION ACID IN SEA URCHIN EGGS

II. EXPERIMENTAL ANALYSIS OF THE PRODUCTION MECHANISM

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INTRODUCTION

In a previous paper (AKETA, 1961) a possibility has been suggested that the breakdown of the cortical granules at fertilization is correlated with the acid production. If it is true, no production of the fertilization acid will be observed in eggs in which breakdown of the cortical granules upon fertilization was artificially inhibited.

The following experiments are attempts to test the above idea.

The author wishes to thank Prof. T. YAMAMOTO and Dr. E. NAKANO for their helpful advice and encouragement. Thanks are also due to the Director of the Sugashima Marine Biological Station, Prof. M. SUGIYAMA and to his staff for making facility for carrying out the work and for the valuable criticism.

EXPERIMENTAL

Eggs of the following four species of sea urchins were used as material: *Hemicentrotus pulcherrimus, Anthocidaris crassispina, Temnopleurus toreumaticus* and *pseudocentrotus depressus.* General procedures for obtaining mature unfertilized eggs and for measuring the pH shift of the egg suspension were the same as described in the previous paper (AKETA, *loc. cit.*). Two means were employed for inhibiting the breakdown of the cortical granules; (a) pretreatment with butyric acid-sea water and (b) narcotization with urethane.

(*a*) *Pretreatment with butyric acid-sea water*

MOTOMURA (1934) found that when the sea urchin egg is treated

with butyric acid-sea water (100 ml. of sea water plus 6 ml. of N/10 butyric acid) for about 10 minutes prior to insemination, formation of the fertilization membrane does not occur upon fertilization. According to him (1941), it is due to inhibition of breakdown of the Janus green granules (cortical granules).

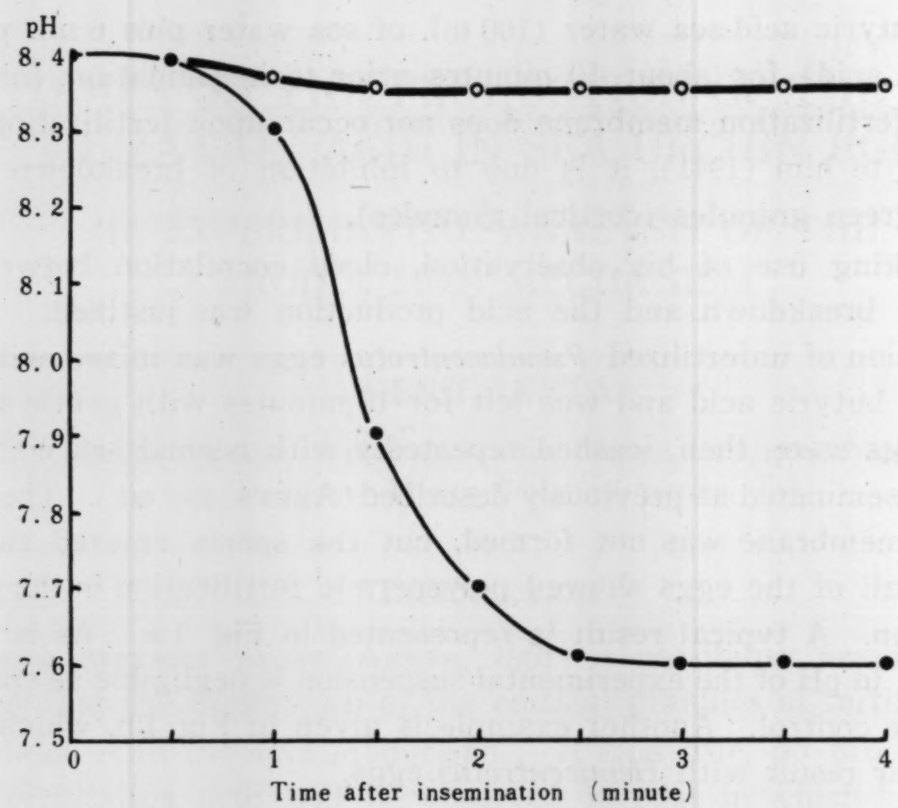
Making use of his observation, close correlation between the granule breakdown and the acid production was justified. 100 ml. suspension of unfertilized *Pseudocentrotus* eggs was mixed with 5 ml. of N/10 butyric acid and was left for 10 minutes with gentle stirring. The eggs were, then, washed repeatedly with normal sea water and were inseminated as previously described (AKETA, *loc. cit.*). The fertilization membrane was not formed, but the sperm entered the egg; nearly all of the eggs showed polyspermic fertilization in the present condition. A typical result is represented in Fig. 1 a. As is shown, the fall in pH of the experimental suspension is negligible as compared with the control. Another example is given in Fig. 1 b, which shows a similar result with *Hemicentrotus* eggs.

(*b*) *Narcotization with urethane-sea water*

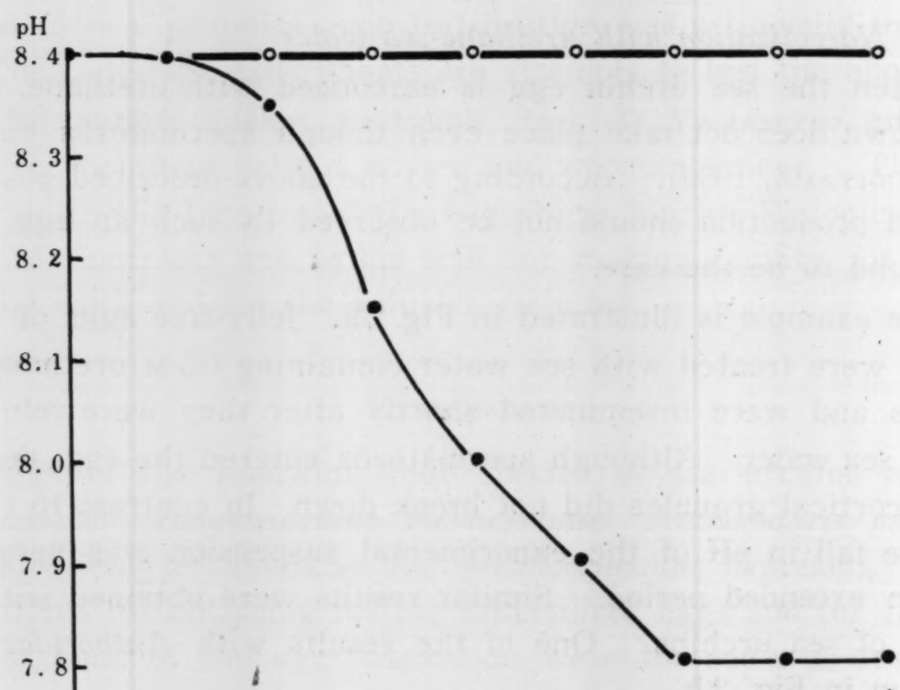
When the sea urchin egg is narcotized with urethane, granule breakdown does not take place even though spermatozoa enter the egg (SUGIYAMA, 1956). According to the above-described possibility, the acid production should not be observed in such an egg, and it was found to be the case.

One example is illustrated in Fig. 2 a. Jelly-free eggs of *Temnopleurus* were treated with sea water containing 0.5 M urethane for 5 minutes and were inseminated shortly after they were returned to normal sea water. Although spermatozoa entered the egg, nearly all of the cortical granules did not break down. In contrast to the control, the fall in pH of the experimental suspension was very slight over an extended period. Similar results were obtained with other species of sea urchins. One of the results with *Anthocidaris* eggs is shown in Fig. 2 b.

It was experienced that, the longer the eggs were narcotized, the less the acid production occurred. In other words, the more the cortical granules broke down, the more the acid was produced. A result which illustrated such a relationship in *Hemicentrotus* egg is given in Fig. 3.



(a)



(b)

Fig. 1 (a and b). Effect of butyric acid-pretreatment on the acid production. 100 ml. suspension of unfertilized eggs was mixed with 5 ml. of 0.1 N butyric acid and left for 10 minutes, followed by the insemination in normal sea water.

●——● control. ○——○ experimental.

(a) *Pseudocentrotus depressus.*

(b) *Hemicentrotus pulcherrimus.*

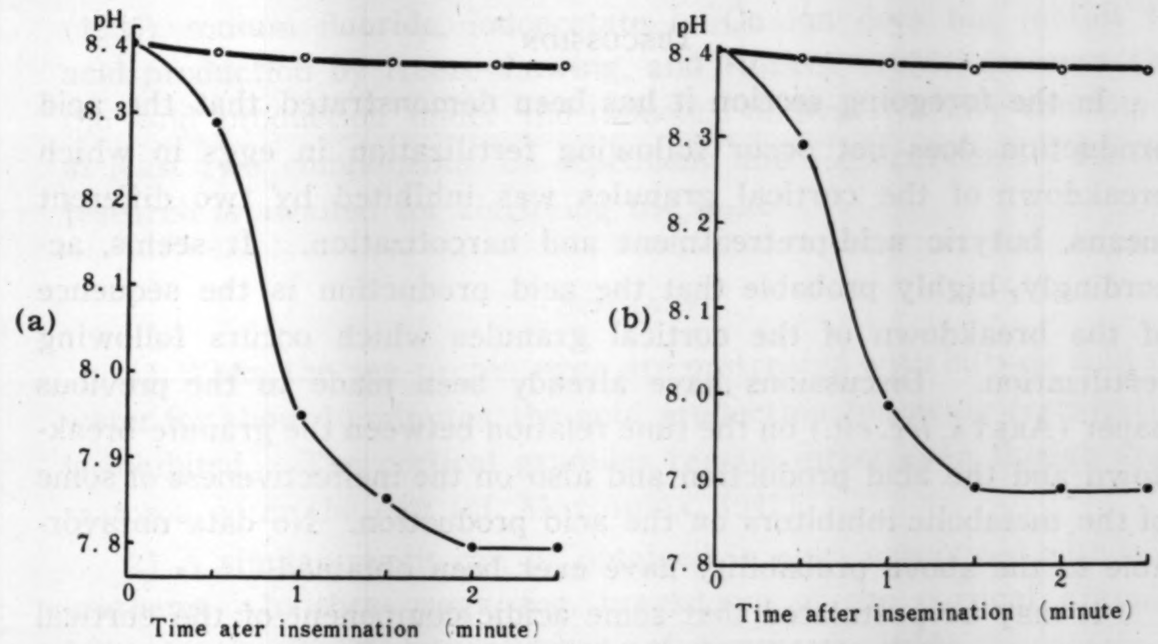


Fig. 2 (a and b). Effect of narcosis on the acid production. Unfertilized eggs were narcotized with 0.5 M urethane and were inseminated after being returned to normal sea water.

●——● control. ○——○ experimental.

(a) *Temnopleurus toreumaticus*. (b) *Anthocidaris crassispina*.

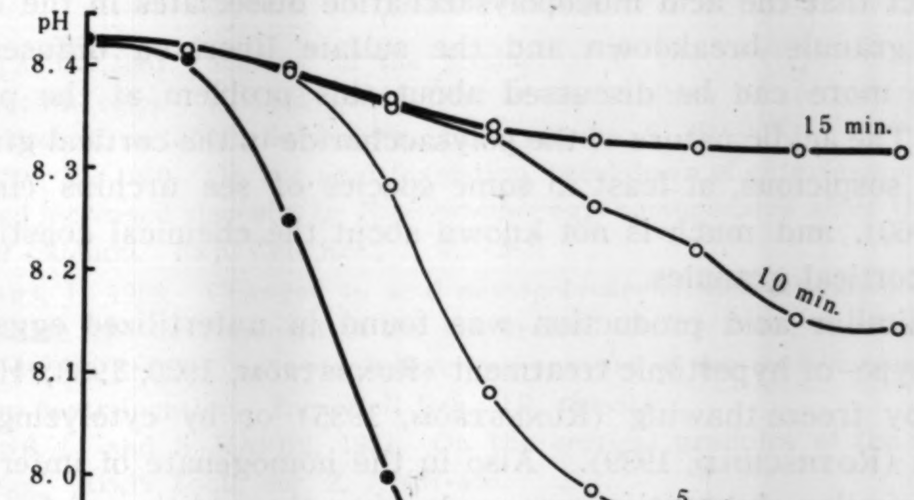


Fig. 3. Relationship between the degree of narcosis and that of the acid production in *Hemicentrotus* eggs. The eggs were treated with 0.5 M urethane for different intervals and were inseminated after being returned to normal sea water.

Discussion

In the foregoing section it has been demonstrated that the acid production does not occur following fertilization in eggs in which breakdown of the cortical granules was inhibited by two different means, butyric acid-pretreatment and narcotization. It seems, accordingly, highly probable that the acid production is the sequence of the breakdown of the cortical granules which occurs following fertilization. Discussions have already been made in the previous paper (AKETA, *loc. cit.*) on the time relation between the granule breakdown and the acid production and also on the ineffectiveness of some of the metabolic inhibitors on the acid production. No data unfavorable to the above probability have ever been obtained.

It may be postulated that some acidic component of the cortical granules is liberated during the process of granule breakdown and diffuses into the surrounding sea water. According to MONNÉ and HÅRDE (1951) cortical granules contain mucopolysaccharide esterified with an acid, presumably sulfuric acid. Even though it seems natural to expect that the acid mucopolysaccharide dissociates in the course of the granule breakdown and the sulfate liberated diffuses out, nothing more can be discussed about this problem at the present time.* The acidic nature of the polysaccharide in the cortical granules became suspicious, at least in some species of sea urchins (IMMERS, 1956, 1960), and much is not known about the chemical constituents of the cortical granules.

A similar acid production was found in unfertilized eggs after either hypo- or hypertonic treatment (RUNNSTRÖM, 1930, 1933; HULTIN, 1950), by freeze-thawing (RUNNSTRÖM, 1935) or by cytolyzing with saponin (ROTHSCHILD, 1939). Also in the homogenate of unfertilized eggs a similar observation was made after the addition of Ca ion or by the treatment with papain (HULTIN, 1950). Although the acid production in these cases may not wholly be alike to each other in nature, it is not improbable that, in some of these cases, the acid production is due partially to breakdown of the cortical granules induced under the experimental conditions. According to RUNNSTRÖM

* Possible bearing of the acid production on the changes in acid mucopolysaccharide is suggested also by RUNNSTRÖM and IMMERS (1956) in the hypothesis presented by them.

(1935) sodium fluoride, iodoacetate or Cu ion does not inhibit the acid production by freeze-thawing, and HULTIN (1950) assumes that the acid production under the influence of hypertonicity consists of at least two components, Ca-dependent and -independent. Further research is awaited for clarifying the issue.

SUMMARY

1) When the sea urchin eggs are pretreated with butyric acid-sea water for about 10 minutes, the acid production following fertilization is inhibited. The cortical granules remain intact even though spermatozoa enter the egg (cf. MOTOMURA, 1941).

2) A similar result can be obtained in eggs narcotized with 0.5 M urethane. In these eggs, too, breakdown of the cortical granules following fertilization is inhibited (cf. SUGIYAMA, 1956).

3) It is deduced that the acid production is caused by the breakdown of the cortical granules which takes place following fertilization.

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EMBRYOLOGIA, Vol. 5, No. 4, pp. 413-422 (March, 1961)

DEFORMITY OF VERTEBRAE INDUCED BY LATHYROGENIC AGENTS AND PHENYL-THIOUREA IN THE MEDAKA (*ORYZIAS LATIPES*)

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INTRODUCTION

Occasional cases have been reported on the occurrence of a deformity in fishes, in which some vertebrae are fused in places. The deformity may have been caused by certain enviromental factors during embryonic life. On the other hand, a mutant "fused" is known in the medaka (*Oryzias latipes*), which is inherited as a simple Mendelian recessive character (AIDA, 1930). WUNDER (1949) reported that a race of the carp (*Cyprinus carpio*) being cultivated in certain districts of Germany has anchylosed vertebrae and that the character is hereditary.

Some years ago, we have collected about thirty wild medaka with fused vertebrae from a certain region of the outskirts of Nagoya. A detailed account of their characteristics and the breeding results of these aberrant medaka will be published elsewhere (YAMAMOTO, TOMITA and MATSUDA, 1961). They proved to be nonheritable. Hence, it has become clear that there are two kinds of vertebral anchylosis in the medaka; hereditary and nonheritable. This fact encourages us to produce such deformity experimentally.

CHANG, WITSCHI and PONSETI (1955) have shown that β-aminopropionitrile and the extract of the sweet pea (*Lathyrus odoratus*), which contains an allied substance, produce dislocation of joints as well as loosening of intersegmental septa in amphibia, and bending of the femur in the chick embryo. Besides β-aminopropionitrile, aminoacetonitrile, methyleneaminoacetonitrile and semicarbazide hydrochloride are known to have similar effect (cf. LALICH, 1958; LEVY, 1959; PONSETI *et al.*, 1958). The phenomenon is known as lathyrism.

The present study reveals that these lathyrogenic agents produce the anchylosis of vertebrae in the medaka. In studying the inhibition of melanin formation, it occurred to us that the embryonic notochord of the medaka can be deformed by treatment with phenylthiourea. This condition results in the fusion of vertebral centra. In association with the fusion of vertebrae and shortening of the body, a significant reduction in number of the anal fin-rays was noted. Effects of the insecticides (parathione and folidol), sodium thiocyanide and *p*-nitrophenol on the notochord development were also studied in addition.

The authors want to express their sincere gratitude to Prof. TOKI-O YAMAMOTO for his constant guidance and encouragement throughout the course of the present experiments, and for critical reading of the manuscript.

MATERIAL AND METHODS

The material used in the present investigation consisted of embryos, fry, and adults of the orange-red and the brown varieties of the medaka (*Oryzias latipes*), an oviparous cyprinodont fish. Eggs were collected in the early morning and reared in Ringer's solution for the medaka (100 parts of M/7.5 sodium chloride, 2 parts of M/7.5 potassium chloride and 2.1 parts of M/11 calcium chloride, pH 7.3 by sodium bicarbonate) as formulated by YAMAMOTO (1939, 1944). They were reared at room temperature. Developmental stages were referred to MATSUI's table of the normal course of development (MATSUI, 1949). At 25° C. the embryos hatch in 10 days. At the end of the first day the egg reaches the late gastrula stage (stage 15). During the second day the formation of eye cup and the differentiation of the myotomes begins (stage 20). The blood circulation is established during the third day (stage 25), and pigmentation of the eye is initiated during the fourth day (stage 26). Movements of the pectoral fins start during the seventh day (stage 29).

One-hundredth molal to M/4,000 aminoacetonitrile, M/100–M/4,000 methyleneaminoacetonitrile, M/100–M/1,000 semicarbazide hydrochloride, M/100–M/1,000 phenylthiourea, M/2–M/20 sodium thiocyanide and M/200–M/2,500 *p*-nitrophenol were dissolved in distilled water and neutralized with phosphate buffer before use. Folidol and parathione were diluted to 1 : 10,000–1 : 200,000 with distilled water before use. The eggs were immersed in these reagents for varying periods, and

then transferred to Ringer's solution which was renewed twice daily.

The effect of sweet pea (*Lathyrus odoratus*) was studied in two ways, *viz.* either by feeding a sweet pea diet to adult fishes and examining their offspring, or by feeding the sweet pea diet to newly hatched larvae and examining them after three months. Dried sweet peas were powdered, and the powder was mixed in amounts up to 75% with the standard diet consisting of two parts of shrimp powder, one part of toasted whole barley flour and small quantity of green tea. In order to avoid a possible effect of unconsumed sweet pea diet, the adult medaka were put into fresh water before spawning.

Results

a) *Aminoacetonitrile and methyleneaminoacetonitrile*

Both chemicals induce a deformity of the notochord. In solutions stronger than M/400 only a few larvae hatch, and these are unable to swim owing to severe deformity. In order to obtain larvae which are able to swim, the embryos must be returned to Ringer's solution at stage 26–28 (beginning of blood circulation). In concentration below M/800 free swimming larvae with deformed notochord can be obtained. At concentrations below M/3,000 the larvae can survive for a considerable period. The development of the circulatory system is scarcely affected in M/400 solution. It seems, therefore, that the inhibitory action of aminoacetonitrile and methyleneaminoacetonitrile on the development of the circulatory system is weaker than that of phenylthiourea as mentioned later. The results are shown in Tables 1 and 2.

Table 1. Effect of aminoacetonitrile on embryos of the medaka*

Concentration	Deformity of notochord	Hatching	Swimming
M/100	+	−	−
M/200	+	±	−
M/400	+	±	±
M/800	+	+	+
M/1,600	+	+	+
M/3,200	±	+	+

* Eggs were immersed in aminoacetonitrile solution from gastrula to hatching.

Table 2. Effect of methyleneaminoacetonitrile on embryos of the medaka*

Concentration	Deformity of notochord	Hatching	Swimming
M/100	+	–	–
M/200	+	±	–
M/400	+	±	±
M/800	+	+	±
M/1,600	+	+	+
M/3,200	±	+	+

* Eggs were immersed in methyleneaminoacetonitrile solution from gastrula to hatching.

In the normal wild medaka, the form index (the ratio of standard length to greatest body depth) is 4.4 (3.6–5.3). This figure is based on measurements of fishes immediately after collection. In the male medaka deformed by treatment with methyleneaminoacetonitrile the index is reduced to 3.2 (2.6–3.8).

It is worth notice that the number of anal fin-rays is remarkably reduced in young and adult fish treated with aminoacetonitrile during embryonic stage. Two individuals in the aminoacetonitrile series have only 12 fin-rays in their anal fins, while the control fish has 16–22 fin-rays.

b) *Semicarbazide hydrochloride*

Semicarbazide also induces deformity of the notochord. In M/200–M/400 solutions the embryos are able to hatch and the fry can swim normally. In these concentrations tissues of non-mesodermal origin are less strongly affected. Eggs immersed from the morula stage until stage 26, and embryos immersed after stage 29, can hatch and the fry have normal appearance. Semicarbazide, therefore, acts chiefly during the period between stage 26 and stage 29. The results are shown in Text-Figure 1 and Table 3.

c) *Sweet pea*

When adult medaka are fed on 75% or 100% sweet pea diets for three months, no visible changes appear in the bone. When just hatched larvae are fed on a 75% sweet pea diet for three months, the fin-rays of all fins show a tendency to bend and become slender

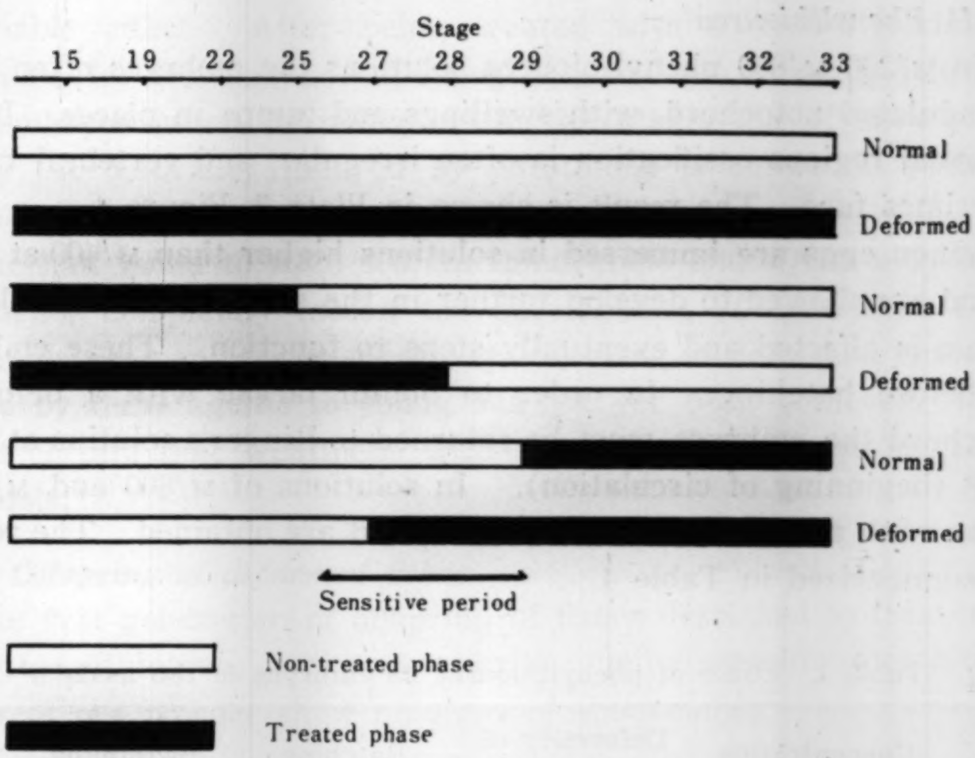

Figure 1. The period of sensitivity to M/200 semicarbazide hydrochloride.

Table 3. Effect of semicarbazide hydrochloride on embryos of the medaka*

Concentration	Deformity of notochord	Hatching	Swimming
M/200	+	+	±
M/400	±	+	+
M/800	−	+	+

* Eggs were immersed in semcarbazide solution from gastrula to hatching.

in form, but the vertebral centra remain normal.

However, adult medaka fed on 75% or 100% sweet pea diets produces offspring which is deformed in about 30%. The deformity seems to be the same as those induced by aminoacetonitrile, methylene-aminoacetonitrile and semicarbazide. There is also a tendency of reduction in number of rays of the anal fin. Two pre-mature fishes have only 11 rays in the anal fin, and one case has only 10 rays (see Plate 7, Figures 2 and 3).

d) *Phenylthiourea*

In M/200–M/800 phenylthiourea solutions the embryos often show an undulated notochord, with swellings and lumps in places. In the abnormal regions ossification is often irregular, and vertebral centra sometimes fuse. The result is shown in Plate 7, Figure 4.

When eggs are immersed in solutions higher than M/400 at stage 15, and are allowed to develop further in the solution, the circulatory system is affected and eventually stops to function. These embryos die before hatching. In order to obtain larvae with a deformed notochord the embryos must be returned to Ringer's solution at stage 26–28 (beginning of circulation). In solutions of M/800 and M/1,000 larvae with a slightly deformed notochord are obtained. The results are summerized in Table 4.

Table 4. Effect of phenylthiourea on embryos of the medaka*

Concentration	Deformity of notochord	Hatching	Swimming
M/200	+	−	−
M/400	+	±	−
M/600	±	+	+
M/800	±	+	+

* Eggs were immersed in phenylthiourea solution from gastrula to hatching.

A significant reduction in number of the anal fin-rays is noted in young and adult fish treated with phenylthiourea during embryonic stage. One individual has only 12 fin-rays in the anal fin.

e) *Folidol, parathione, sodium thiocyanide and p-nitrophenol*

The deformity induced by these chemicals is severe, and becomes apparent in early stages. Most embryos die before hatching. The body size is reduced, the differentiation of eyes is abnormal, and the circulatory system is incomplete. On the other hand, the notochord is little affected. In solutions of lower concentrations, in which the embryos are able to hatch, no visible malformations appear.

In 1 : 10,000 folidol or parathione solution at stage 10, 24 or 28, most embryos die within 24 hours. In 1 : 50,000 solution they die within 36 hours. Solutions of a concentration of 1 : 200,000 have no

appreciable effect. After being treated with 1 : 10,000 folidol or parathione for six hours, and being returned to Ringer's solution, some embryos hatch normally, while the others die of severe deformity. A deformity induced by parathione is shown in Plate 7, Figure 5.

One half molal to M/20 sodium thiocyanide and M/200–M/2,500 *p*-nitrophenol completely inhibit the development of embryonic body without showing any tissue specificity. Rearing of deformed embryos induced by these agents to young has not yet been achieved. It is not yet certain that treatment with these agents induces vertebral anchylosis.

f) *Offspring of deformed fishes*

The first generation of offspring of fishes deformed by treatment with phenylthiourea, aminoacetonitrile, methyleneaminoacetonitrile and sweet pea powder show no signs of abnormality.

Discussion

Deformity of the vertebral centra in the medaka occurs rarely in nature. So far, lathyrogenic agents are known to cause dislocation of joints, loosening of intersegmental septa, bending of femur and formation of knobs of bone in Amphibia and in the chick embryos. These agents are known to have highly tissue specific effect, the mesodermal tissues being mostly affected. Our findings indicate that lathyrogenic agents cause deformity of the embryonic notochord and the anchylosis of vertebrae.

In the present investigation phenylthiourea was found to induce the same deformity in the medaka, but it was shown that it also severely affects the development of the circulatory system. The agent has a general teratogenic effect on the trunk region of the body. In lower concentrations, in which the embryos develop until hatching, the effect is noticeable only in the notochord.

A number of other chemicals, folidol, parathione, sodium thiocyanide and *p*-nitrophenol completely arrest the development of the body without showing specific deformity of the notochord.

The region of the deformity in the notochord may depend on the stage of treatment. Embryos treated with M/200 phenylthiourea, M/200 aminoacetonitrile or M/200 methyleneaminoacetonitrile at the

morula stage and returned to Ringer's solution at stage 25 develop normally. When left in the teratogenic solution until stage 26–27, the anterior part of the notochord becomes abnormal; when left in the solution during the whole course of development, the embryos do not hatch, and the deformity spreads over the entire length of the notochord. Embryos reared in these solutions after stage 29 show no notochord deformities. Larvae with notochord deformity restricted to the posterior part are not obtained.

In the offspring of adult fishes fed on sweet pea powder the region of the deformity is irregular. This suggests that in this case the teratogenic factor may act at various stages of development. The factor may be contained in the yolk and released at various stages.

Semicarbazide is apparently the most rapidly penetrating agent among the various agents used. Therefore, the determination of the phase of maximal sensitivity for this agent is comparatively easy. It was found that the critical period is between stage 26 and stage 29. Since the teratogenic agents used are applied at a stage preceding the onset of ossification, they act apparently not directly on the bone formation, but rather on the formation of preosseous matrix.

It is worth notice that in association with anchylosis of vertebrae and shortening of the body length, the number of the anal fin-rays is remarkably reduced. In the normal medaka it is 16–22 and in the genetic "fused" 13–18 (YAMAMOTO, personal communication). Among the treated fishes, six pre-mature individuals were encountered which had only 10–12 rays in the anal fin. As would be expected, the deformity induced by these teratogenic agents was found to be non-heritable.

SUMMARY

1) Treatment of embryos of the medaka (*Oryzias latipes*) with lathyrogenic agents such as aminoacetonitrile, methyleneaminoacetonitrile, semicarbazide and sweet pea powder induces deformity of the notochord, anchylosis of vertebrae and shortening of the body.

2) Phenylthiourea is found to produce nearly similar effects.

3) The insecticides, parathione and folidol exert a drastic inhibition of embryonic development with little specific effect on the notochord. The same is true of *p*-nitrophenol and sodium thiocyanide.

4) In parallel with the fusion of vertebrae and shortening of the body, the anal fin-rays are significantly reduced in number.

5) The induced deformity is proved to be nonheritable.

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Explanations of Plate 7

Figure 1. From the top: normal male, deformed male treated with methyleneaminoacetonitrile, deformed male treated with aminoacetonitrile.

Figure 2. A deformed male among the offspring of an adult fed on sweet pea powder. Anal fin with 10 rays.

Figure 3. The anal fin of the same animal. Note the papillar processes.

Figure 4. Embryo treated with M/200 phenylthiourea solution. Note the undulated notochord.

Figure 5. A rare case of deformity induced with parathione. Most of these embryos die before hatching. Note the notochord with a small lump.

Figure 6. A defomed fry induced with M/200 semicarbazide hydrochloride. Note the notochord with hump.

Figure 7. A deformity induced with semicarbazide hydrochloride. Fry in the stage of the onset of ossification.

Fig. 1

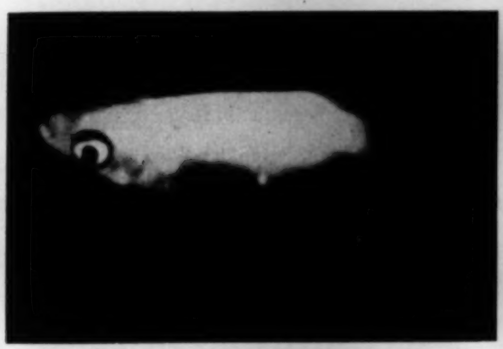

Fig. 2

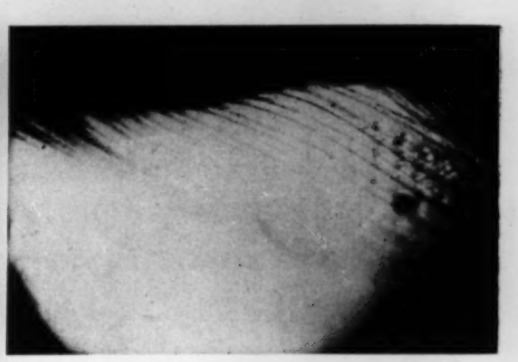

Fig. 3

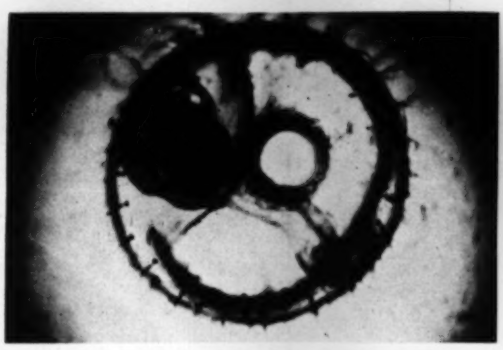

Fig. 4

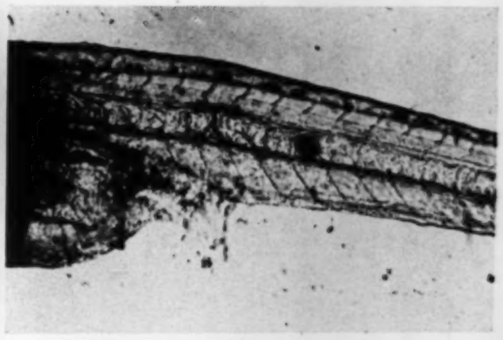

Fig. 5

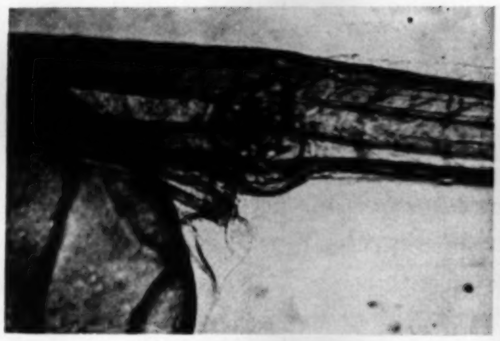

Fig. 6

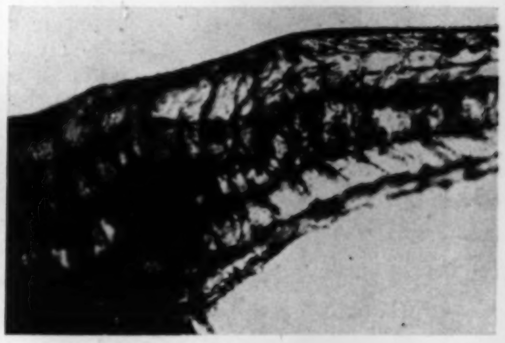

Fig. 7

EMBRYOLOGIA, Vol. 5, No. 4, pp. 423-439 (March, 1961)

ON THE PHENOL OXIDASE OF EMBRYONIC AND LARVAL STAGES OF THE MEDACA (*ORYZIAS LATIPES*)[1][2]

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INTRODUCTION

The mechanism of melanin formation has attracted much attention, and many reports on this problem have been published (DAWSON and TARPLEY 1951, LERNER 1953, MASON 1955). Phenol oxidases such as tyrosinase and dopa oxidase are generally assumed to play a part in the process of melanin formation. Tyrosinase has been demonstrated to occur in some invertebrates and vertebrates and universally in plant sources. Previously the authors of this paper have succeeded in demonstrating the presence of tyrosinase and dopa oxidase in the skin of the medaka (HISHIDA, TOMITA and YAMAMOTO 1961). Quantitative study on the activities of tyrosinase and dopa oxidase was further made in the skin of some color varieties of the medaka (TOMITA and HISHIDA 1961). It is natural to expect that embryos, in which melanin synthesis proceeds very actively, possess high tyrosinase and dopa oxidase activities. The study of tyrosinase synthesis during development is also important for the problem of genetic control of melanin formation for which the genes *B* and *b* are responsible.

Therefore, the present paper is devoted primarily to studies of nature of these phenol oxidases and the synthesis of these enzymes during embryonic development. In addition, the effects of chemical inhibitors on melanin formation and on tyrosinase synthesis are studied.

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1) This is a part of the "Phenogenetic studies on color varieties of the medaka (*Oryzias latipes*)" being carried out under the direction of Prof. TOKI-O YAMAMOTO at the Biological Institute, Faculty of Science, Nagoya University.

2) We express our deep gratitude to Dr. SEIZO KATSUNUMA, former President of Nagoya University for his suggestion on this project and the continued encouragement during the studies.

MATERIAL AND METHOD

The material used are eggs and larvae of the two varieties of the medaka (*Oryzias latipes*), the orange-red (*bR*), and the brown (*BR*).

A tiny cluster of eggs is carried by the female at her vent after spawning. They are removed by a wide-mouthed pipette. For the experiments, eggs were washed repeatedly with distilled water and were separated from each other. This procedure is necessary to avoid low oxygen tension and to get synchronous development. Then, eggs were allowed to develop in the isotonic RINGER's solution for the medaka composed of M/7.5 NaCl 100 parts, M/7.5 KCl 2 parts, M/11 $CaCl_2$ 2.1 parts (pH 7.3 being adjusted by N/10 $NaHCO_3$) as formulated by YAMAMOTO (1939, 1944) until desired stages were reached at room temperature (25–28°C). The numerical designation of the stages is adopted following MATUI's table of normal development (1949) throughout the development. At 25°C the embryos hatch in 10 days. Eye-cup formation and differentiation of somites begin during the second day (stage 20). The pigmentation of the eye begins during the fourth day (stage 26). Movements of the pectoral fins start during the seventh day (stage 29). For the details of the daily development, MATUI's table (1949) or RUGH's book (1948) should be consulted.

The embryos at various stages were homogenized in distilled water with the usual type of glass homogenizer, and were centrifuged at 1,000 r.p.m. for 10 minutes. The supernatant was used as the crude enzyme. For measuring dopa oxidase activity 100–300 eggs per Warburg vessel and for measuring tyrosinase activity 500–1,000 eggs were used. Endogenous respiration was reduced by adding either monoiodoacetic acid or iodoacetamide in the final concentration of M/100. These reagents have been shown to have little or no effect on dopa oxidase and tyrosinase activities of the medaka (TOMITA and HISHIDA 1961). In other cases, endogenous oxygen consumption was eliminated by adding alcohol to the crude enzyme solution up to a final concentration of 70%, at which concentration cytochrome oxidase is destoryed (HESSELBACH 1951). By such treatment tyrosinase and dopa oxidase activities are reduced to some extent owing to denaturation.

Enzymatic activity was measured in terms of oxygen consumption at 30°C., using a Warburg mamometer. The center well contained 0.1 ml. of 20% KOH, the main part of the vessel contained 0.5 ml. of M/10 phosphate buffer (pH 6.8) and 1.0 ml. of the enzyme solution, and the side arm contained 0.5 ml. of the substrate. A parallel control experiment was run without substrate. The substrate solutions used in the phenol oxidase assay were 0.1% d.l. tyrosine in 0.08% sodium carbonate, 0.1% dopa, 0.5% *p*-, *m*-, and *o*-cresols, 0.5% phenol, 0.5% *p*-chlorophenol, 0.5% *p*-bromophenol, 0.5% *p*- and *m*-nitirophenols, 0.5% 3, 4- and 3, 5-dimethylphenols, and 0.5% catechol.

In order to obtain the embryos entirely free from melanin pigment and to investigate the effect of chemical inhibitors on the enzyme synthesis, series of RINGER's solutions containing various concentrations of hydroquinone, hydroquinone-monobenzylether, phenylthiourea, thiourea, thiouracil, methyl-

thiouracil, *o*- and *m*-cresolindophenols, *m*-aminophenol, and *m*-phenylenediamine respectively, were used as culture media.

To test the effect of metal ions on melanin formation, $HgCl_2$(10^{-2}–10^{-7} M), $ZnCl_2$(10^{-2}–10^{-4} M), $MnCl_2$(10^{-2}–10^{-4} M), $Co(NO_3)_2$(10^{-2}–10^{-4} M), and $CuSO_4$(10^{-3}–10^{-7} M) solutions were also used.

Histochemical tests for tyrosinase and dopa oxidase were performed by the method similar to that used in the study of isolated scales (HISHIDA, TOMITA and YAMAMOTO 1961). Larvae were pretreated by immersion for one minute either in 10% neutral formalin or in 50% ethanol at 0°C. The treated larvae were incubated in dopa or tyrosine solution at 30°C., together with antibiotics such as streptomycin, and penicillin, or iodoacetamide. The results were examined after an incubation period of 5 hours for dopa, and of 24 hours for tyrosine.

Other experimental procedures will be described in the relevant sections.

RESULTS

a) Nature of the phenol oxidase extracted from the medaka embryos

Phenol oxidase from the embryos was capable of oxidizing tyrosine and dopa, but it did not oxidize other phenols, such as *p*-, *m*-, and *o*-cresols, phenol, catechol, *p*-chlorophenol, *p*-bromophenol, *p*- and *m*-nitrophenols, 3, 4- and 3, 5-dimethylphenols. Therefore, specificity of phenol oxidase in the embryos is strictly restricted to tyrosine and dopa, a situation found previously in the adult fish (TOMITA and HISHIDA 1961).

In the embryos of brown variety, the embryonic melanophores first appear on the yolk sac when several somites are formed in the embryonic body (stage 20). However, no phenol oxidase activity was detected at this stage. This may be ascribed to very low activity of the enzyme. In the orange-red variety, only a few slightly pigmented melanophores are found on the yolk sac even in later stages, the melanin being localized mainly in the eyes. In both the brown and the orange-red varieties the amount of melanin pigment increases considerably a few days before hatching. The tyrosinase and dopa oxidase were first detected at stage 26, when melanin begins to accumulate in the eyes. Then, as the pigmentation proceeds in the eyes and the peritoneal covering, the activities of both enzymes increase linearly until hatching, the activity of dopa oxidase being nearly ten times higher than that of tyrosinase. It should be noted that the ratio of both activities remains almost constant throughout the development. The difference in dopa oxidase activity between

the brown and the orange-red varieties was shown to be negligible. The results are shown in Figure 1.

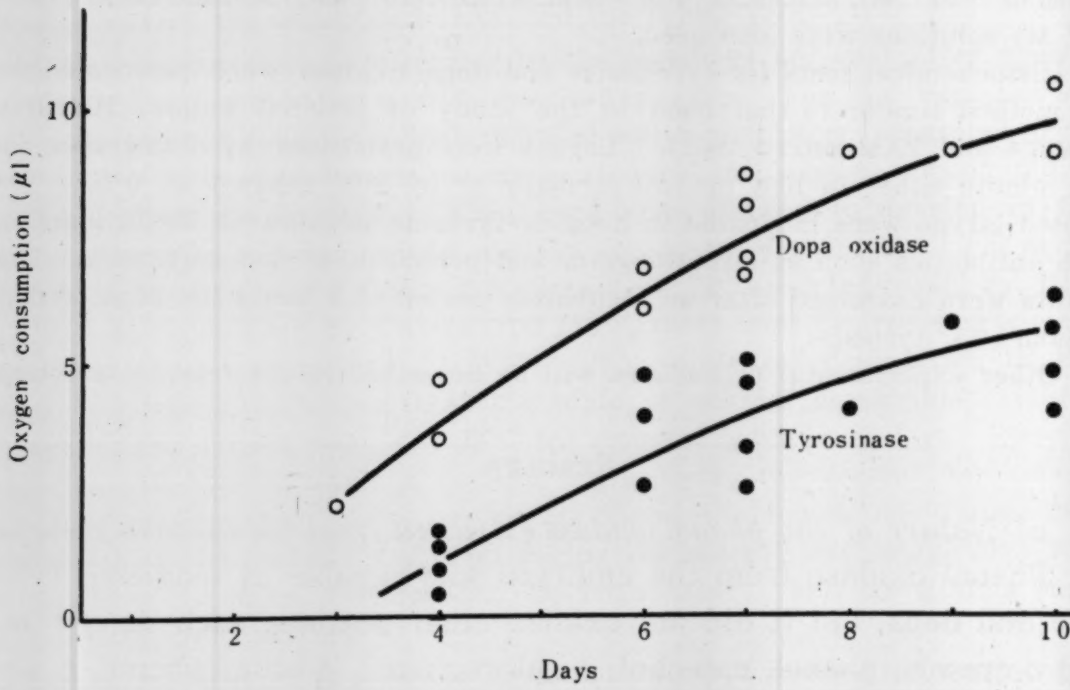


Fig. 1. Activities of dopa oxidase and tyrosinase of homogenates of embryos of the orange-red variety. Enzymatic activities are expressed in oxygen consumption per hour per 100 eggs (dopa oxidase) or per 500 eggs (tyrosinase), in the presence of substrate (endogenous oxygen consumption is subtracted).

In order to decide whether tyrosinase and dopa oxidase are bound to the melanin granules, homogenates of embryos of the orange-red variety were fractionated by centrifugation at 3,000 r.p.m. for 20 minutes. KCl (M/7.5), NaCl (M/7.5) and sucrose (M/3.8) solutions were used as media for the fractionation. Dopa oxidase activity was demonstrated in the melanin-free supernatant rather than in the precipitate containing the melanin granules. The conclusion can be drawn that dopa oxidase activity is mainly localized in the melanin-free supernatant. However, for a final answer to this question it would be necessary to repeat the experiment with embryos of the brown variety, which has no amelanotic melanophores. Unfortunately, it was difficult to obtain sufficient number of eggs of the brown variety.

b) *Effect of metal ions*

It is generally held that phenol oxidase is a copper protein.

Although no direct evidence is obtainable as yet, it may be assumed that tyrosinase and dopa oxidase of the medaka are also copper proteins. Endogenous oxygen consumption of crude enzyme preparations from the medaka was shown to increase on addition of copper ions. Copper-free crude enzyme, obtained by treatment with M/50 sodium cyanide and subsequent dialysis against M/100 phosphate buffer for 24 hours, showed increase of oxygen consumption on addition of copper ions, though the formation of black pigment was not noticed.

The effects of metal ions on melanin formation were also tested *in vivo*. The metal ions examined were shown to be toxic to embryos at high concentrations. In surviving embryos melanin formation was not visibly affected by metal ions such as Hg(10^{-6} M), Zn(10^{-3} M), Mn(10^{-3} M), and Co(10^{-3} M). Copper ions (10^{-5}–10^{-6} M) did not accelerate melanin formation *in vivo*.

c) *Effect of phenylthiourea, thiourea, thiouracil, and methylthiouracil*

It seems that tyrosinase is synthesized to synchronize with melanin synthesis in the eye and the peritoneal covering. But dopa oxidase activity is detected at an earlier stage than tyrosinase activity because of high activity of the former. The question naturally arises as to whether tyrosinase and dopa oxidase are a single enzyme or not. If each activity is attributable to a separate enzyme, a single activity would appear during the course of development in the media containing specific inhibitors. The next question is whether, in the artificial albino produced by exogenous inhibitors, tyrosinase is synthesized at all, or only the protein moiety of tyrosinase free from copper is synthesized.

In order to solve these questions simultaneously, two types of inhibitors, those which combine with copper of the enzyme, *i.e.* phenylthiourea, thiourea, thiouracil, and those which interfere with tyrosine-dopa system, *i.e.* hydroquinone, hydroquinone-monobenzylether, were added to culture media. Phenylthiourea, hydroquinone, and hydroquinone-monobenzylether were found to induce artificial albino larvae during immersion in these solutions.

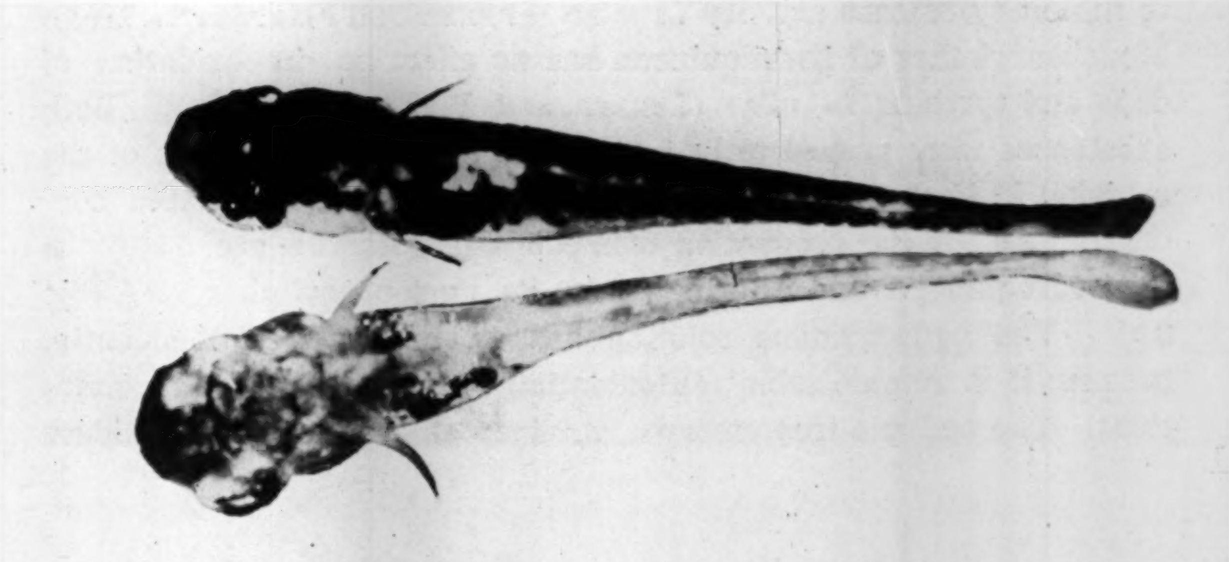
Embryos cultured in phenylthiourea solution higher than M/3,000 from an early stage were wholly devoid of melanin pigment, and in

M/8,000, a trace of pigment appeared only in the eye and head regions. When the pigment-free embryos, which were cultured in phenylthiourea solution till stage 26, were transferred into RINGER's solution, the melanin pigment soon developed to the same extent as in the case of embryos cultured only in RINGER's solution. The "albino" larvae induced by phenylthiourea, gradually produced the pigment in RINGER's solution, and after three weeks, a considerable amount of pigment appeared in the eye and head regions. Phenylthiourea treatments frequently induced a retardation in development and morphological defects, such as lack of the swim bladder.

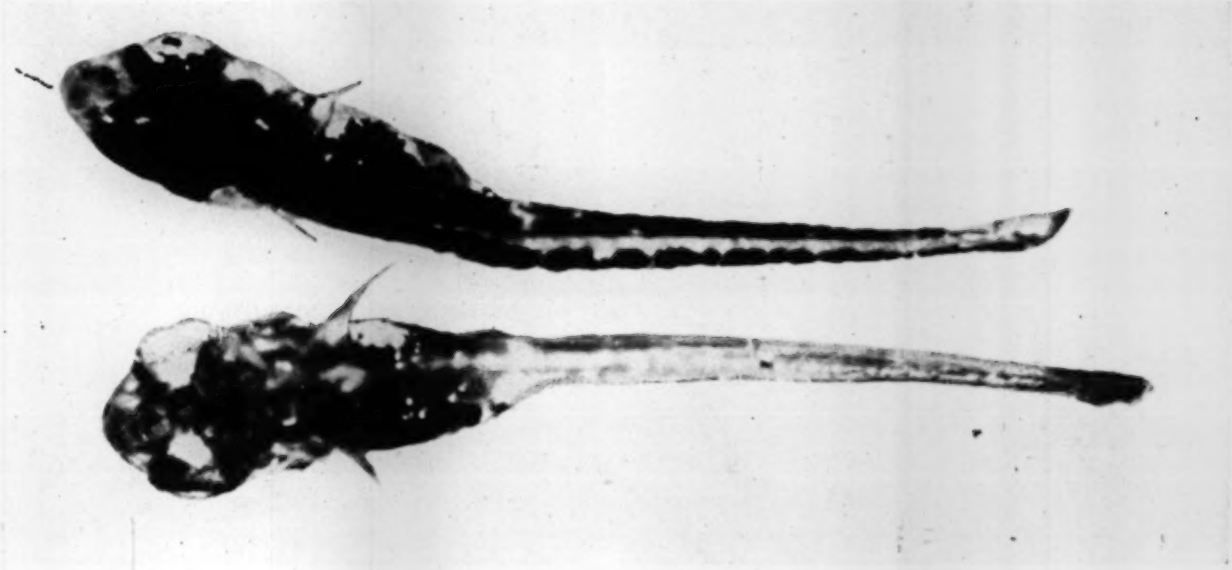
Thiourea also induced artificial albinism in concentrations higher than M/4, but the embryos died before hatching. In concentrations lower than M/20, thiourea had no effect on melanin formation. Thiouracil and methylthiouracil in nearly saturated solutions (M/200 and M/400 respectively) showed no effect on melanin formation.

Thus, phenylthiourea is the most powerful agent for inducing artificial albinism. When tested on both the brown and the orange-red varieties of the medaka, there was no difference in effect at a concentration of M/3,000, melanin formation being completely suppressed in both varieties (see Fig. 2 *a*). However, in concentrations ranging from M/6,000 to M/7,000 the brown variety showed formation of some melanin in the eyes, whereas the orange-red variety still remained without pigment. In a concentration of M/8,000 considerable amounts of melanin appeared in the eyes in the brown variety whereas only some melanin was formed in the orange-red variety. Here, quantitative melanin determination would yield more clear-cut results.

Homogenates of the embryos which had been cultured in phenylthiourea solution of M/3,000 from an early stage, had no dopa oxidase activity in any stage. These were dialyzed against M/100 phoshate buffer for 2 days but no enzymatic activity was noted. It is clear from the experiments on the controls cultured in RINGER's solution that dialysis against M/100 phosphate buffer itself is not inhibitory to dopa oxidase. Furthermore, to preclude the possibility that a trace of phenylthiourea, still remaining in the preparation might exert an inhibitory effect on the enzyme, adhesion of phenylthiourea to the dialyzed preparation was tested by the effect upon plant tyrosinase. But the preparation had no inhibitory action. The preparation was washed well in the cold with 70% alcohol in which phenyl-



a



b

Fig. 2. *a.* The effect of phenylthiourea treatment; normal larva (above) and artificially induced albino larva (below) of the brown variety (*BR*). *b.* The effect of hydroquinone-monobenzylether treatment; normal larva (above), and artificially induced albino larva (below) of the brown variety (*BR*).

thiourea is more soluble than in buffer, but the activity was not detected as before. These experiments favor the view that tyrosinase is not synthesized in phenylthiourea solution.

d) *Effect of hydroquinone and its monobenzylether*

Hydroquinone is known to accelerate the oxidation of dopa, but

to inhibit tyrosinase activity *in vitro* (LERNER and FITZPATRICK 1953). Monobenzylether of hydroquinone has no effect on the oxidation of dopa and tyrosine *in vitro* (LERNER and FITZPATRICK 1953). Both substances were proved to be harmful to embryos and larvae of the medaka, at least in higher concentrations. The most suitable concentrations for the production of melanin-free larvae are 5×10^{-4} M for hydroquinone, and 2.5×10^{-6} M for its monobenzylether (see Fig. 2 *b*). The hydroquinone solution has to be renewed frequently, because it is autoxidizable (autoxidation is indicated by red coloration). The melanin-free embryos obtained showed low dopa oxidase

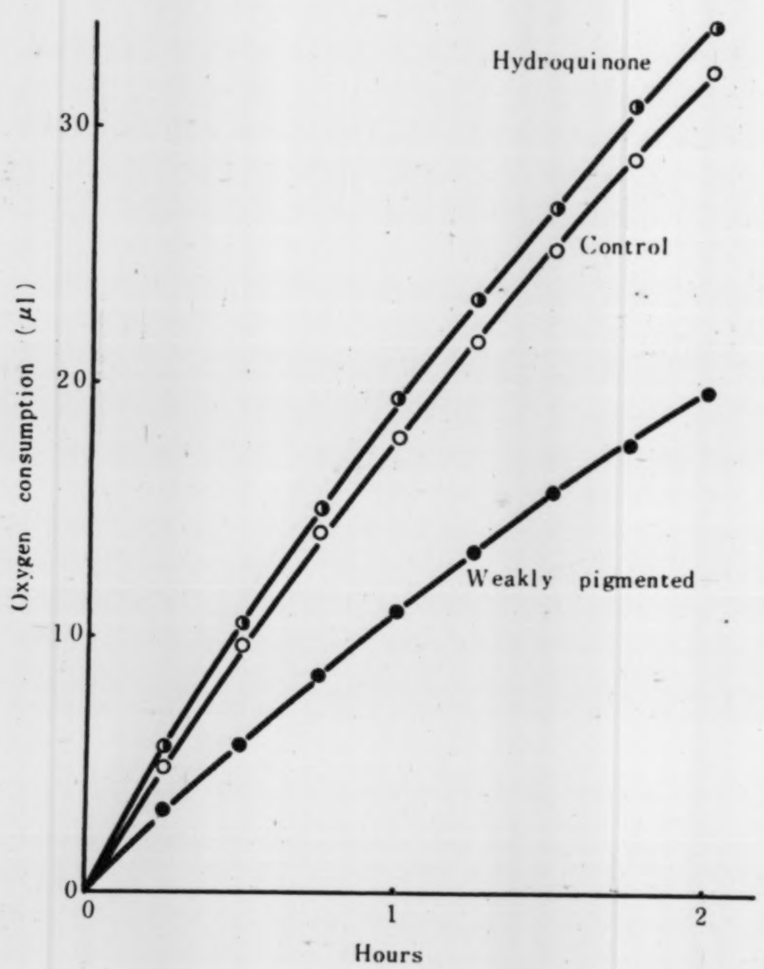


Fig. 3. Dopa oxidase activity of homogenates of weakly pigmented embryos induced by treatment with 5×10^{-4} M hydroquinone. Enzymatic activity is expressed in oxygen consumption per 250 eggs. The curve "hydroquinone" represents dopa oxidase activity of normal embryo extracts in the presence of 5×10^{-4} M hydroquinone.

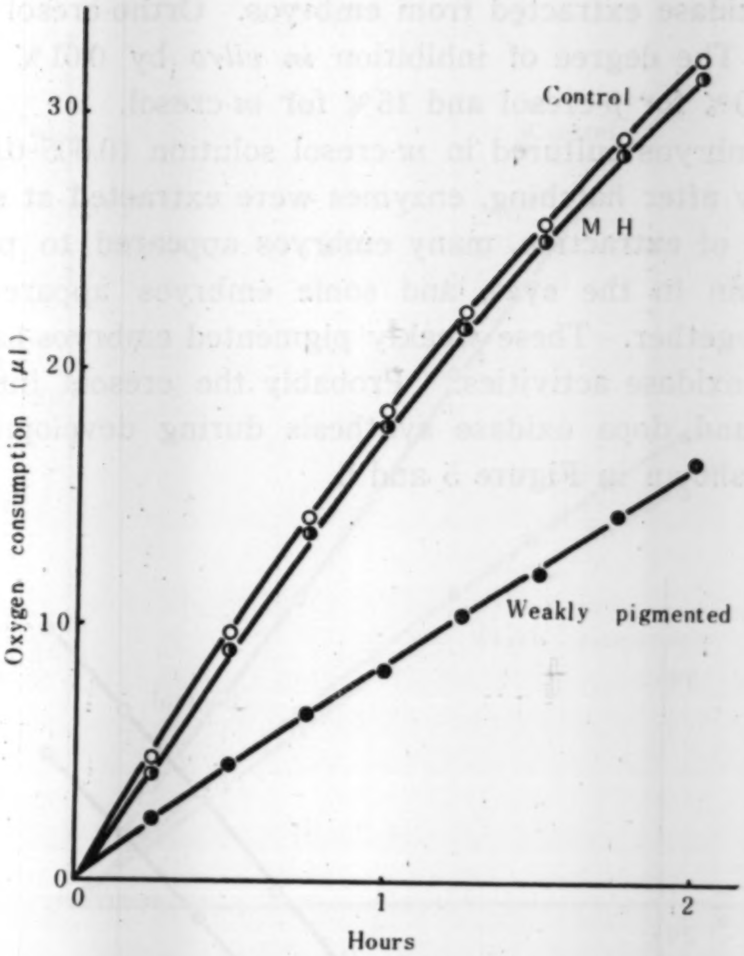


Fig. 4. Dopa oxidase activity of homogenates of weakly pigmented embryos induced by treatment with 2.5×10^{-6} M monobenzylether of hydroquinone. Enzymatic activity is expressed in oxygen consumption per 250 eggs. The curve "M.H" represents dopa oxidase activity of normal embryo extracts in the presence of 2.5×10^{-6} M monobenzylether of hydroquinone.

activity, notwithstanding the fact that 5×10^{-4} M hydroquinone was shown to accelerate dopa oxidation *in vitro*. The results are shown in Figures 3 and 4.

e) *Effect of cresols*

Embryos cultured in *p*- and *m*-cresol solutions in concentrations ranging from 0.005 to 0.01% often lost their melanin pigment. They also showed malformations and their development was retarded. All embryos died before hatching in concentrations above 0.01%. *In vitro*, *p*- and *m*-cresols were shown to inhibit the activity of tyrosinase

and dopa oxidase extracted from embryos. Ortho-cresol had hardly any effect. The degree of inhibition *in vitro* by 0.01% cresol solutions was 40% for *p*-cresol and 15% for *m*-cresol.

Since embryos cultured in *m*-cresol solution (0.005–0.01%) had a low viability after hatching, enzymes were extracted at stage 30–33. At the time of extraction, many embryos appeared to possess only some melanin in the eyes, and some embryos apparently lacked melanin altogether. These weakly pigmented embryos had only very low phenol oxidase activities. Probably the cresols interfere with tyrosinase and dopa oxidase synthesis during development. The results are shown in Figure 5 and 6.

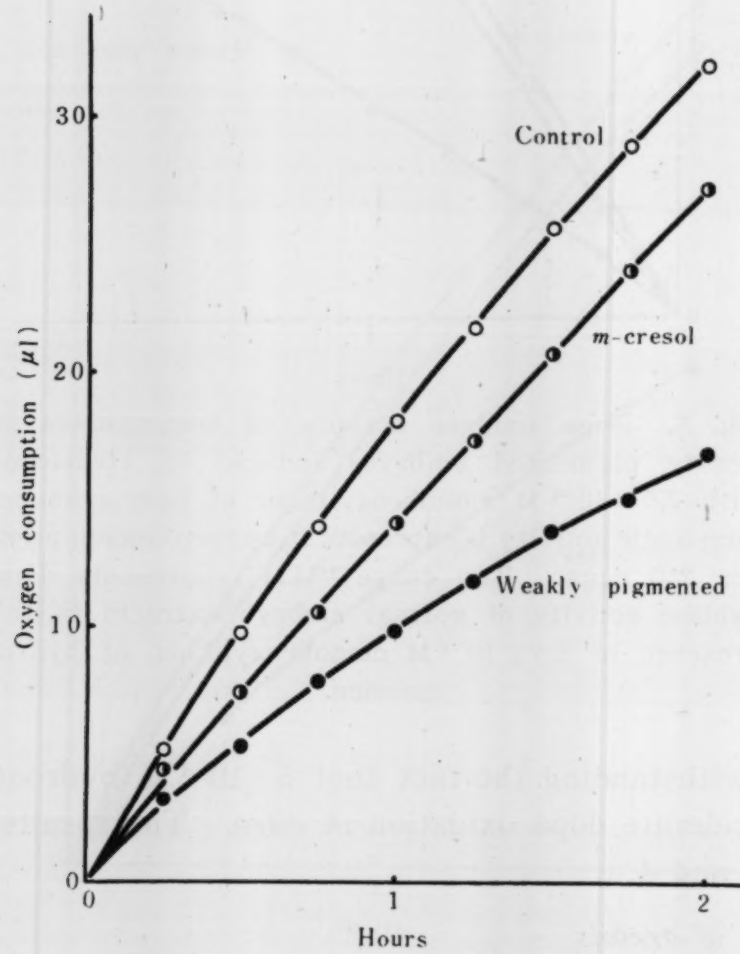


Fig. 5. Dopa oxidase activity of homogenates of weakly pigmented embryos induced by treatment with 0.005% *m*-cresol. Enzymatic activity is expressed in oxygen consumption per 250 eggs. The curve "*m*-cresol" represents dopa oxidase activity of normal embryo extracts in the presence of 0.005% *m*-cresol.

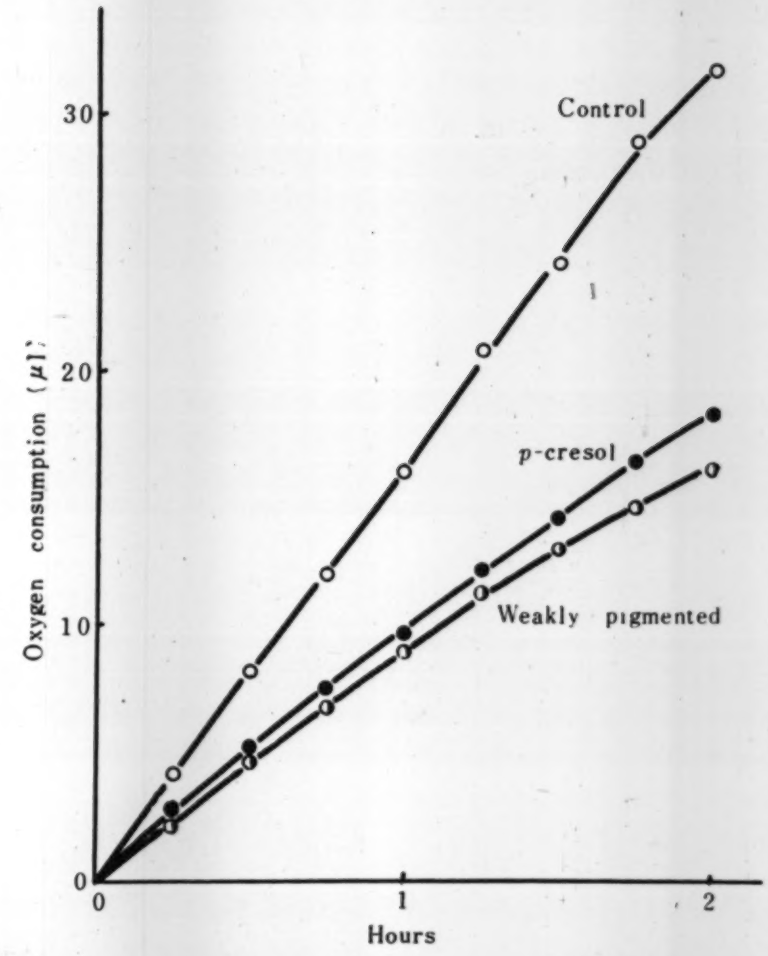
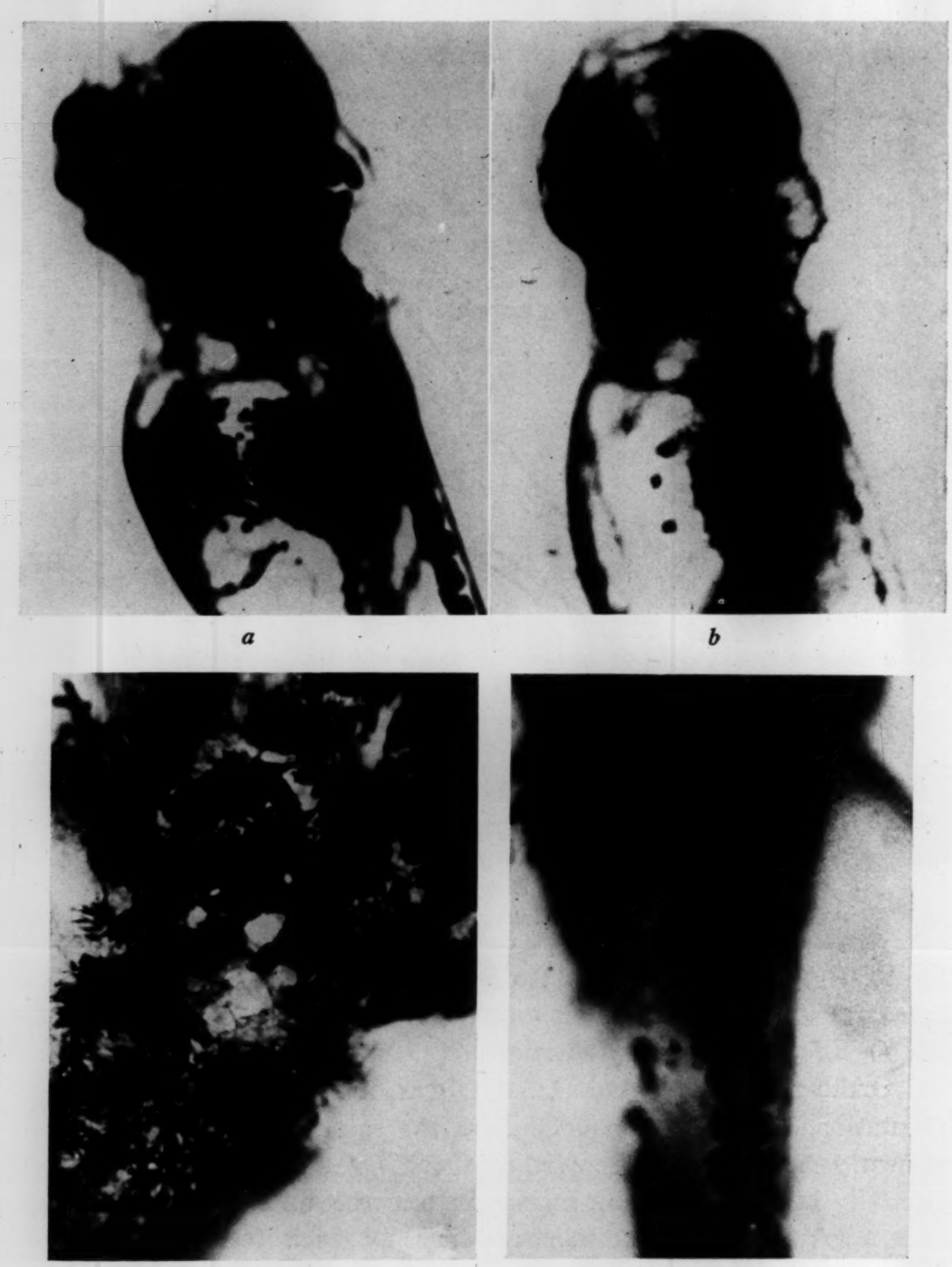


Fig. 6. Dopa oxidase activity of homogenates of weakly pigmented embryos induced by treatment with 0.005% *p*-cresol. Enzymatic activity is expressed in oxygen consumption per 250 eggs. The curve "*p*-cresol" represents dopa oxidase activity of normal embryo extracts in the presence of 0.005% *p*-cresol.

f) *Effect of other inhibitors*

Ortho-cresolindophenol (1:5,000) was found to induce low pigmentation. Meta-cresolindophenol at the same concentration (which is nearly the saturation) showed no effect. Meta-aminophenol (M/500) partially inhibited melanin formation, but true albinism occurred only in a few cases. Para-aminophenol has not been tested because of its toxicity. Meta-phenylenediamine (M/1,000) induced low pigmentation. Para- and ortho-phenylenediamines are autoxidizable, and hence unsuitable.

Up till now no agents were found which inhibit only the differentiation of melanophores without interfering with melanin formation.



a b

c d

Fig. 7. *a*. Larva of the brown variety (*BR*). Fully developed melanophores are present, and mask the guanophores.——*b*. Larva of the orange-red variety (*bR*). The "amelanotic" melanophores which contain little melanin pigment are not visible. The guanophores are seen as dots.——*c*. Tyrosinase test in larva of the orange-red variety (*bR*). Fully developed black melanophores are present.——*d*. Dopa oxidase test in larva of the orange-red variety (*bR*). Fully developed black melanophores are present.

g) Histochemical detection of tyrosinase and dopa oxidase in larvae of the orange-red variety

The hatched fry were not tested for enzymatic activities quantitatively, since it was not easy to collect sufficient material. Instead, tyrosinase and dopa oxidase activities were cheked histochemically. It was found that, on incubation with dopa or tyrosine solution with iodoacetamide, "amelanotic" melanophores of the orange-red variety turn black and assume almost the same shape as the black melanophores of the brown variety (see Figure 7).

Discussion

As far as the substrate specificity of phenol oxidase is concerned, it is known that enzymes prepared from invertebrates and plants oxidize various phenols, while those from vertebrates (except the amphibia) catalyze the oxidation of tyrosine and dopa only (Lerner 1953, Dawson and Tarpley 1951). It has now become clear that phenol oxidase from embryos and larvae of the medaka can oxidize only tyrosine and dopa as that from the adult fish does (Tomita and Hishida 1961).

It is clear that tyrosinase and dopa oxidase activities appear in the embryos of the medaka at the stage of beginning of melanin formation. These enzymes are synthesized in the course of development and their activities increase gradually, corresponding closely with melanin formation in the embryos. Phenols which are not oxidized by phenol oxidase from the medaka show an inhibitory action on tyrosinase and dopa oxidase activities *in vitro*. and inhibit the synthesis of the enzymes *in vivo*. Amphibian phenol oxidase oxidizes *p*-cresol and *m*-cresol, but it does not oxidize *o*-cresol. Ortho-cresol is usually not oxidized by phenol oxidases, except by those from some plant sources (Dawson and Tarpley 1951). Para-cresol exerts a stronger inhibition on melanin formation of embryos of the medaka than *m*-cresol. Ortho-cresol has no inhibitory effect at all.

No significant difference in dopa oxidase activity can not be detected between the brown and the orange-red varieties. This may be due to the reason that the embryos are tested as a whole including the eye and the peritoneal covering the melanin synthesis of

which is far stronger than that of the yolk sac and on the surface of the embryonic body. Moreover, activities of dopa oxidase and tyrosinase in adult skins of the orange-red variety are higher than those in the brown variety, contrary to what we first expected (TOMITA and HISHIDA 1961). In the present study, dopa oxidase of the embryos of the orange-red variety are demonstrated to localize in the melanin-free supernatant as well as in the precipitate. These facts support the view that fully melanized granules no longer contain active dopa oxidase and tyrosinase.

Thiourea inhibits melanin formation *in vivo* in concentrations higher than M/4. Phenylthiourea completely inhibits melanin formation in the embryos and induces "albino" larvae at a concentration of M/3,000. In embryos in which synthesis of melanin pigment has already begun, it inhibits further pigmentation and phenol oxidase synthesis.

In concentrations ranging from M/6,000 to M/7,000, the embryos of the brown variety are able to form some melanin pigment in the eye, whereas those of the orange-red variety still remain without pigment. These facts afford the first and valuable evidence in support of biochemical differences in melanin formation between the brown (*BR*) and the orange-red (*bR*) varieties.

The inhibitory effect of phenylthiourea and thiourea on phenol oxidase can be achieved by chelating the copper ion which is essential for phenol oxidase action. It is known that these agents depress the function of thyroid gland and that thyroid hormones regulate the pigmentation of feather in bird (WILLIER 1952), but the concentrations in which these are effective as such are much lower. It may therefore be that these agents directly inhibit the process of melanin formation but not indirectly through thyroid function. In fact, these agents inhibit completely both activities of dopa oxidase and tyrosinase in extracts of embryos of the medaka, probably combining with copper as described in other sources (LERNER 1953). Melanin-free embryos induced by phenylthiourea exhibit neither dopa oxidase activity nor tyrosinase activity. Meta-cresol which inhibits both activities of dopa oxidase and tyrosinase *in vitro,* also inhibits the synthesis of both enzymes.

On the other hand, hydroquinone inhibits the activity of tyrosinase but not that of dopa oxidase. Monobenzylether of hydro-

quinone, which may *in vivo* decompose to hydroquinone, has no inhibitory effect on dopa oxidase and tyrosinase activities *in vitro* (LERNER 1953). The embryos cultured in adequate concentrations of hydroquinone or its monobenzylether exhibit albinism, and show low activities of both tyrosinase and dopa oxidase. Thus, these agents which *in vitro* do not inhibit dopa oxidase activity, cause *in vivo* a great decrease in the activity. For this fact there are two possible explanations: either the two enzymes are synthesized in the same process, which is inhibited by hydroquinone or its benzylether, or tyrosinase and dopa oxidase are in reality one and the same enzyme, at least in the medaka.

The effects of metal ions and inhibitors for melanin formation on the embryos were studies by visual observation only, for lack of an appropriate quantitative method of melanin determination. Therefore, slight differences in effect may have escaped observation. Particularly some metal ions may not have been entirely without effect.

OKA (1931) studied the development of the melanophores in embryonic and larval stages of the medaka. He held that normal cell size and melanin formation are due to a dominant gene *B*, whereas the recessive gene *b* would cause the development of small size melanophores and the production of a smaller amount of melanin. In the present study, the presence of tyrosinase and dopa oxidase in the larvae of the oranged-red variety was histochemically demonstrated. The size and shape of "amelanotic" melanophores of the orange-red variety proved to be nearly the same as those of fully developed black melanophores in the brown variety. It should be noted that in the larvae of the orange-red variety, tyrosinase and dopa oxidase present *in vivo* in an inactivate state.

SUMMARY

1) Phenol oxidase extracted from embryos of the medaka (*Oryzias latipes*) can oxidize tyrosine and dopa only. The enzyme is gradually synthesized in the course of development, which synchronizes with melanin formation in the eye and the peritoneal covering.

2) Copper-free enzyme, obtained by treatment with M/50 KCN and subsequent dialysis against M/100 phosphate buffer, shows

increase of oxygen consumption on addition of copper ions, though the formation of black pigment is not noticed.

3) Addition of chemical inhibitors such as, phenylthiourea, thiourea, hydroquinone-monobenzylether, hydroquinone, and *p*- and *m*-cresols to the culture media, induces artificially weak pigmentation in embryos. Embryos cultured in phenylthiourea have neither tyrosinase activity, nor dopa oxidase activity. Both activities of embryos cultured in other inhibitors are, although present, far lower than those of normal animals. On return to normal medium, however, melanin formation is gradually recovered in the brown variety.

4) A significant difference in critical concentrations of phenylthiourea for inducing artificial albinism is noted between the brown and the orange-red varieties, although no significant difference in both activities of tyrosinase and dopa oxidase is detected.

5) Larvae of the orange-red variety possess "amelanotic" melanophores which contain only a small amount of melanin. Their shapes are nearly the same as those in the brown variety.

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